A NEW PREPARATION OF BILIVERDIN

by

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INTRODUCTION.

Biliverdin is closely related to the bile pigment-iron compounds which are intermediates of the bile pigment formation from haemoglobin, and which occur in catalase. A convenient method for its preparation is, therefore, desirable.

The only method hitherto available (Lemberg, 1932, 1934) is based on the oxidation of bilirubin by ferric chloride in glacial acetic acid. The formation of by-products in this process necessitates a complicated and wasteful purification, so that the yield of pure product is only 20–30 p.c. of the bilirubin.

Malloy and Evelyn (1937) have published an oxidation method for the determination of bilirubin. By the oxidation of bilirubin in ethyl alcohol with various oxidizing agents they obtain a stable blue colour, which is the basis for their estimation with the photoelectric colorimeter. Concerning an intermediate green colour that is obtained during the oxidation, they state that "the occurrence of an isosbestic point (at 522μ) supports the contention of Peterman and Cooley (1933) that the green colour of so-called biliverdin is in reality a mixture of yellow bilirubin and blue bilicyanin in varying proportions". This claim is based solely on spectrophotometric evidence.

Lemberg (1934) had found, on the contrary, that the blue oxidation stage of the Gmelin reaction is not due to a single compound ("bilicyanin") but to a mixture of biliverdin with a violet higher oxidation product ("biliviolin" or, as Siedel calls this class of bile pigments, "bilipurpin"). We have therefore reinvestigated the oxidation of bilirubin under the conditions employed by Malloy and Evelyn, and have found not only that biliverdin is formed but that this oxidation affords the best preparation of biliverdin so far known.

EXPERIMENTAL WORK.

Crystalline bilirubin prepared from ox gall stones was used as the starting material. One litre of ethyl alcohol containing 10 c.c. concentrated hydrochloric

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acid and 2 c.c. of 100 vol. p.c. hydrogen peroxide was added to a solution of 40 mg. bilirubin in 200 c.c. chloroform. This was allowed to stand at room temperature. After a few minutes the solution turned green; after an hour the solution, though blue-green, still contained some bilirubin. This could be seen if a small part of the alcoholic solution were taken into ether and extracted with a little 2 p.c. hydrochloric acid. Biliverdin goes into the aqueous layer, and bilirubin remains in the ether solution.

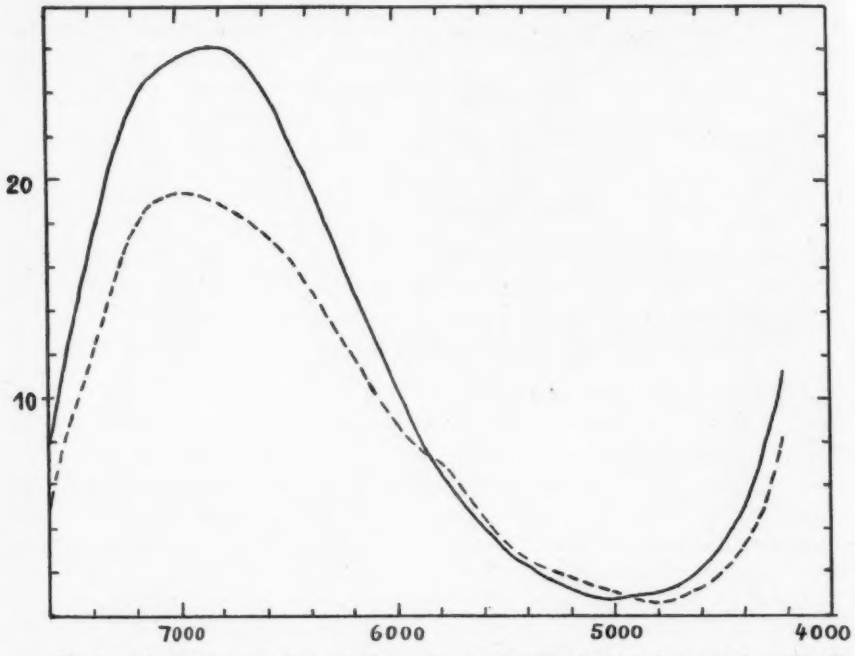

Fig. 1. Absorption spectrum of biliverdin (continuous line) and of "end product" (interrupted line).

Abscissa $= \lambda$ in Å.
Ordinate $= \epsilon$ mol $\times 10^{-3}$.

After the solution had stood for 90 minutes no bilirubin was left. At this stage the solution was a green-blue colour, and its absorption spectrum is shown in Fig. 1 (continuous line). It can be seen that the absorption curve does not show any indications of biliviolins which have, in acid solution, an absorption band at about 5900Å. The maximum at 6800Å corresponds to the position of the absorption maximum of biliverdin hydrochloride. A comparison with the molar extinction coefficient of biliverdin hydrochloride at this wavelength (ϵ mol 6800Å

= 28,000) shows that, to judge from the absorption, the yield of biliverdin in this reaction is 95 p.c. From this solution crystalline biliverdin can be obtained as described below.

In order to examine the further course of the reaction, an additional 1 c.c. of 100 volumes p.c. hydrogen peroxide was added to the alcohol solution and the reaction was allowed to proceed for 16 hours. The colour of the solution during this time changes relatively little, from a greenish blue to a pure blue. Fig. 1 (interrupted line) shows the absorption of the solution after 16 hours. The absorption spectrum has also undergone rather small changes: the maximum in the red is lower, and has been shifted from 6800 to 7000 Å, while in the region between 5600 and 5800 Å there is slightly increased absorption. The major part of the absorption is due to a biliverdin type of bile pigment, while the small increase in the green part of the spectrum is probably due to a slight formation of biliviolins.

In spite of this similarity of absorption, however, the compound is now no longer biliverdin, since it cannot be extracted from ether by 2 p.c. hydrochloric acid. We have been unable to isolate this new, weakly-basic bile pigment with biliverdin-like absorption. Taken into ether it was oxidized by the excess of hydrogen peroxide to a biliviolin. Its properties make it appear likely that the further oxidation has not altered the tetrapyrrolic system, the resonance of which determines the absorption, but has attacked the unsaturated side chains of biliverdin.

PREPARATION OF BILIVERDIN.

In the preparation of pure biliverdin by this method then, it is necessary firstly to choose the length of action of the oxidizing agent so that biliverdin is the main oxidation product, and secondly to remove the verdin from the ether as quickly as possible in order to avoid further oxidation.

100 mg. of pure[2] crystalline bilirubin are dissolved in 100 c.c. of boiling chloroform and traces of undissolved pigment are removed by filtration. An equal volume of ethyl alcohol containing one p.c. hydrochloric acid is added and the solution is at once cooled under the tap to a temperature of 20°C. One c.c. of 100 volumes p.c. hydrogen peroxide is added dropwise with shaking and the mixture is left standing. During one hour it turns from yellow to green with a yellow tinge, finally to a green-blue. From time to time small samples are withdrawn and examined for the presence of bilirubin as described above. The reaction is stopped when the ether solution remains only slightly yellow. It is not advisable to proceed further, since the complete oxidation of bilirubin is immediately followed by further oxidation of biliverdin and the complete separation of biliverdin from its oxidation product is more difficult that its separation from bilirubin. With a room temperature of 20°C. this point was reached after 1–1½ hours standing; at 30° the reaction took only 15 minutes, but is more difficult to control.

On addition of 800–1,000 c.c. of ether and shaking with 2 p.c. hydrochloric acid, most of the biliverdin goes into the aqueous phase as biliverdin hydrochloride and precipitates in green flocks, which are collected by filtration. The last remainders of biliverdin are extracted by 20 p.c. hydrochloric acid. It is advisable to bring the pigment from this extract immediately back into 200 c.c. of ether with sodium acetate solution, and to extract biliverdin hydrochloride once more with 2 p.c. hydrochloric acid; a little blue pigment remains in the ether.

[2] We have observed that some samples of bilirubin are impure in spite of being crystalline. Such samples give a much lower yield of biliverdin.

From the collected acid solutions biliverdin is brought back with sodium acetate solution into 800 c.c. of ether containing 50 c.c. of glacial acetic acid. The flocks of biliverdin hydrochloride are dissolved in glacial acetic acid and added to this mixture. Biliverdin is taken up by the ether with deep greenish-blue colour. Flocks which come out in the inter-layer are once more dissolved in a few c.c. of glacial acetic acid, added to 100 c.c. of fresh ether and once more shaken with the aqueous layer obtained in the first extraction. After one more repetition of this procedure practically all biliverdin is found in the ether layer. The greater part of the glacial acetic acid is now washed away with dilute sodium acetate solution; and the ether solution, without separation from some biliverdin which has come out, is concentrated by distillation to 30 c.c. Most of the biliverdin is thus precipitated. The remainder is precipitated from the ether mother liquor by washing with sodium acetate solution and water. Finally all the collected biliverdin, including that which may stick to the walls of the flasks, is dissolved in hot methyl alcohol and crystallizes from it in blue-green needle clusters. Yield: 40 mg.

SUMMARY.

The "blue stable stage" in the oxidation of bilirubin by hydrogen peroxide in acid alcoholic solution (method of Malloy and Evelyn) is biliverdin (dehydrobilirubin).

A new method for the preparation of biliverdin from bilirubin based on this yields about 40 p.c. of pure crystalline product.

Prolonged oxidation by hydrogen peroxide does not attack the tetrapyrrolic nucleus but the unsaturated side chains of biliverdin. This is not accompanied by pronounced changes of the absorption spectrum.

REFERENCES.

Lemberg, R. (1932): Lieb. Ann., 499, p. 25.
Lemberg, R. (1934): Biochem. J., 28, p. 978; Chemistry & Industry, 53, p. 1024.
Malloy, H. T. and Evelyn, K. A. (1937): J. biol. Chem., 122, p. 597.
Peterman, E. A. and Cooley, T. B. (1933): J. lab. & clin. Med., 19, pp. 723, 743.

STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

2. THE ISOLATION OF THREE STRAINS OF *RICKETTSIA BURNETI* FROM THE BANDICOOT *ISOODON TOROSUS.*

by

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The present paper reports the finding of three bandicoots (*Isoodon torosus* Ramsay) actively infected with Q fever.

The three bandicoots belonged to a series of 109, the liver, spleen and kidney of which were inoculated into guinea-pigs. Six guinea-pigs, mostly very young ones, did not survive the inoculation and are excluded from the series. Of the 103 bandicoots, 60 were captured on the mainland, nearly all in the Nambour district, and one of them was found infected; 43 came from Cowan Cowan on Moreton Island, and two were infected.

The successfully inoculated guinea-pigs and the subsequent passages of the three strains of *R. burneti* are set out in Table 1. Charts of the animals of the first three passages are illustrated in Fig. 1.

The guinea-pigs primarily inoculated from bandicoots 52 and 129 remained afebrile for ten days. Inoculation of their livers and spleens caused obvious infection in the second passage animals. With the third strain obvious infection began with the primary guinea-pig.

The first strain was continued for three passages of obvious infection, the other two strains for five passages. Two points in the passaging (see Table 1) call for explanation. In the third passage of Strain II, the guinea-pig J590 was inoculated in the belief that it was a Q-immune pig. When however it reacted with a typical fever its history was reviewed, and it was found not to have been infected with Q fever previously. In the fifth passage of Strain III one of the new guinea-pigs J761, developed an inapparent infection only. There was no significant fever, but the animal was later found to be immune. With these exceptions each strain behaved typically; the infected animals developed a characteristic fever and were rendered immune to the stock laboratory strain of Q fever; Q-immune guinea-pigs remained afebrile after inoculation with the bandicoot strain.

Each bandicoot strain was inoculated into mice, and characteristic rickettsiae were demonstrated in their livers and spleens.

Certain information about the three infected bandicoots is given in Table 2.

TABLE 1.

Passaging of the Three Bandicoot Strains in Guinea-pigs.

	Passages.					
1	2	3	4	5	6	Immunity Test.
A484— →	A505+ →	A540+				A540 immune
		A541+ →	A569+			A569 immune
		*B414—	*J315—			
		*J290—				
I		4 Mice†				
A941— →	A981+ →	J724+				J724 immune
		J590+				J590 immune
		J723+ →	J739+			J739 immune
		J591—	J738+ →	J749+		J749 immune
			*J661—	J748+ →	J763+	J763 immune
II			*J668—	*A934—	J764+	J764 immune
				*A935—	*J621—	
					*J624—	
					4 Mice†	
A973+ →	J718+					J718 immune
	J717+ →	J732+				J732 immune
	*A885—	J731+ →	J747+			J747 immune
	*A887—	*J626—	J746+ →	J761—		J761 immune
III		*J629—	*A923—	J762+		J762 immune
			*A924—	*J626—		
				*J602—		
				4 Mice†		

\+ Febrile reaction.
— No febrile reaction.
† Rickettsiae seen in stained smears of liver or spleen (usually both).
* Immune guinea-pigs.

TABLE 2.

The Infected Bandicoots.

Serial Number of Bandicoot.	Source.	Date of Capture.	Sex.	Weight Grams.	Spleen. Weight Grams.	Spleen. Percentage of Body Weight.	Serum Agglutination of *R. burneti.* 1:10	1:30	1:100	1:300
52	Nambour	July, 1938	M	2,173	24	1·10	+++	—	—	
129	Cowan Cowan	March, 1939	F	835	1·9	0·23	+++	+++	+++	±
139	Cowan Cowan	March, 1939	M	650	2·3	0·35	+++	+++	+++	

The serum of each bandicoot was found by Miss Freeman to agglutinate *R. burneti*. The spleen of bandicoot 52 was considerably enlarged, with hyperplasia of the pulp largely obscuring the Malpighian bodies. The spleens of the other two bandicoots were normal in size.

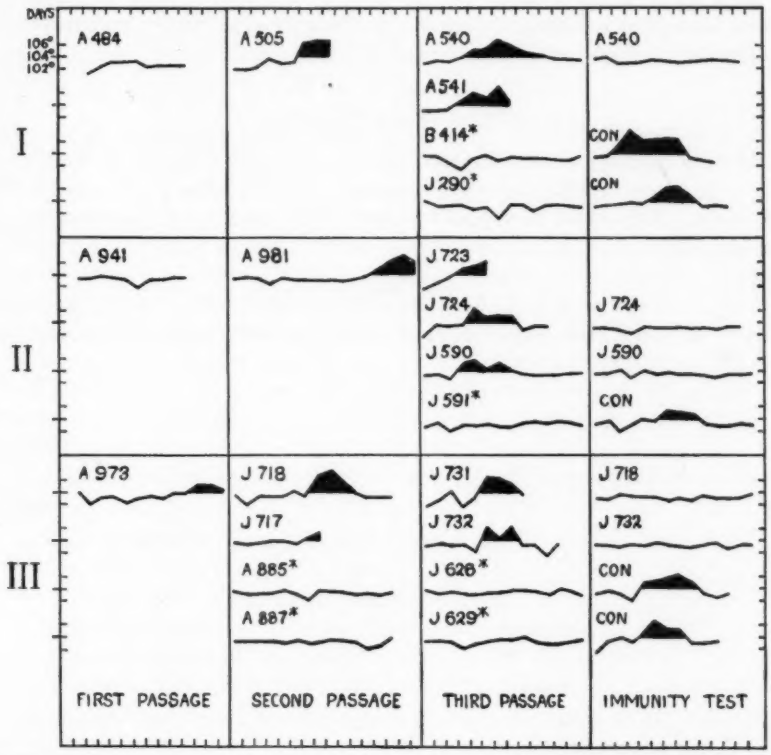

Fig. 1. Charts of some of the guinea-pigs used in the isolation of the three bandicoot strains of *R. burneti*.

* = Immune controls.
CON = Non-immune controls.

Bandicoot 52 was, at the time of its receipt in the laboratory, supporting about 70 nymphal scrub ticks (*Ixodes holocyclus*) and 11 fleas (*Pygiopsylla species*). Guinea-pigs inoculated with these parasites did not become infected. Bandicoot 129 carried three male *Haemaphysalis humerosa* which did not infect an inoculated guinea-pig. The *H. humerosa* on bandicoot 139, however, were infected with Q fever, and from them was established the fifth tick strain reported in the previous paper.

SUMMARY.

Three bandicoots (*Isoodon torosus*) have been discovered naturally infected with Q fever.

This confirms the finding, already described, of agglutinins for *Rickettsia burneti* in the sera of captured bandicoots (Derrick *et al.*, 1939), and further establishes the bandicoot as a natural reservoir of Q fever.

ACKNOWLEDGMENTS.

We are grateful to Sir Raphael Cilento, Director-General of Health and Medical Services, Queensland, for permission to publish this paper, and to Miss M. Freeman, Walter and Eliza Hall Institute, Melbourne, for the bandicoot agglutination tests.

REFERENCE.

Derrick, E. H., Smith, D. J. W., Brown, H. E. and Freeman, M. (1939): Med. J. Austral., 1, p. 150.

STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

3. THE TRANSMISSION OF Q FEVER BY THE TICK *HAEMAPHYSALIS HUMEROSA.*

by

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The tick *Haemaphysalis humerosa* Warburton and Nuttall (1909) has been shown to be a natural vector of Q fever among the native animal population (Smith and Derrick, 1940). Following this discovery, an investigation into the relations prevailing between *Rickettsia burneti*, the causative agent of Q fever, and its arthropod host was undertaken with the dual purpose of shedding further light upon its own relationship to other members of the Rickettsia Group, and the possible importance of *H. humerosa* as an immediate agent in the production of human infections.

The data presented in this paper may conveniently be treated in the following sections:

1. The Infection of Larval, Nymphal and Adult Ticks.
2. Transfer of the Virus from Larva to Nymph.
3. Transfer of the Virus from Nymph to Adult.
4. Possible Transfer of the Virus from Adult to Larva.
5. Distribution and Multiplication of Rickettsiae within the Tick.
6. The Infectiousness of Tick Tissues and Tick Faeces.

Transmission experiments were conducted using guinea-pigs as experimental animals. When fed upon the host animals the ticks were contained in gelatin capsules supported by an elastic bandage—a modification of the method described by Jellison and Philip (1933). Capsules of one-quarter ounce fluid capacity, made for veterinary use, were chosen. The closed end of a capsule was cut off and the body slit longitudinally into a number of strips which were bent outwards radially. The prepared capsule was pushed through a small hole in a strip of elastic bandage and applied to the depilated skin of the host. A narrow strip of bandage inside the capsule prevented young ticks from entering the crevice between the capsule wall and the body of the guinea-pig. Observation and manipulation of ticks upon the host animals were easily accomplished by slipping off the caps of the feeding cells.

1. *The Infection of Larval, Nymphal and Adult Ticks.*

The ticks used in these experiments were bred from wholly or partially engorged females collected from bandicoots. The partially fed ticks were allowed to

complete their engorgement upon guinea-pigs. After the completion of oviposition the ticks were dissected, examined for rickettsiae by means of smears, and inoculated into guinea-pigs. None of the ticks thus examined was found to be infected; no rickettsiae were detected in smears of their organs; neither did they infect the animals into which they were inoculated. A number of the progeny of each tick were examined in a like manner. In several instances the young ticks were reared to maturity by successive feeds upon guinea-pigs. No evidence of the presence of Q fever infection in these ticks was obtained.

The mode of infecting the ticks was to feed them upon infected guinea-pigs during the febrile period. The subsequent immunity of the host guinea-pigs, in addition to abundant evidence from collateral experiments, was in each case indicative of the infectivity of the blood taken up by the feeding ticks. Larval, nymphal and adults ticks were successfully infected in this manner.

Larval Ticks.

Larval ticks were fed upon infected guinea-pigs in batches ranging from approximately twenty to one hundred ticks at intervals of approximately ten, twenty, thirty-eight and eighty days after hatching (Table 1). The young ticks readily attached to the host animals and became fully engorged within forty-eight hours, after which they were removed.

No rickettsiae were seen in smears of five quiescent larvae which were examined, three on the fourth and two on the eighth day after engorgement. No rickettsiae were detected in serial sections of a sixth tick examined on the fourth day after feeding. However, the virus was recovered from batches of six ticks inoculated into guinea-pigs on the eighth and fourteenth days, but not on the fourth day, after the completion of feeding upon infective blood. Most of the larvae were allowed to develop further and were examined for infectivity in their later instars, as described later, when about half were found infected.

Nymphal Ticks.

Batches of nymphal ticks were exposed to infection at intervals of approximately eleven, seventeen, thirty-one and forty-two days after metamorphosis. The ticks became fully engorged and were removed within forty-eight to ninety-six hours after being placed on the host animals. Fifteen days after engorgement five nymphae were inoculated into a guinea-pig with a negative result. The rest of the ticks were examined after their metamorphosis into adults when thirty-one out of fifty-six were found infected.

Adult Ticks.

A number of adult ticks with attendant males were fed upon two infected guinea-pigs during the febrile period. The ticks were transferred to a second pair of hosts to complete their engorgement during the febrile period of the latter animals. Ticks were fed thus at intervals of approximately seventeen and thirty-two days after metamorphosis. At the completion of oviposition, twenty to thirty-five days after engorgement, the virus was recovered from six of eight female ticks examined by guinea-pig inoculation. In the case of three of four ticks dissected and smeared before inoculation, large numbers of rickettsiae were seen. All four ticks infected the animals into which they were injected. None of the male ticks was examined.

Four male ticks removed from a bandicoot were placed upon an infected guinea-pig two days prior to the beginning of fever. They were removed three days after the six-day febrile reaction of the guinea-pig, during which period they were noted to be attached to and feeding upon the host. The ticks were dissected, smears were prepared, and they were then inoculated

into a single guinea-pig. No rickettsiae were seen in the smears and the guinea-pig failed to become infected.

The data relevant to the infection of the ticks are summarized in Table 1. Ticks of each batch infected are designated by the terms Stock A, Stock B, etc.

TABLE 1.

The Infection of Larval, Nymphal and Adult Ticks.

Origin of Ticks.		Infection of Ticks.			
Host Bandicoot Serial No.	Progeny of Tick Serial Number	Instar Infected.	Days after Hatching or Metamorphosis.	Stock.	Remarks.
150	A27	Larva	10	C	Became completely engorged within forty-eight hours during the febrile period of the infected host guinea-pigs.
74	A2	Larva	20	A	
176	A40	Larva	38	I	
		Larva	80	B	
101	A15	Nymph First Generation	11	D	Became completely engorged after forty-eight to ninety-six hours feeding during the febrile period of the infected host guinea-pigs.
108	A16		42		
150	A28	Nymph Second Generation	17	G	
128	A29	Nymph	31	E	
136	A30				
101	A15	Adult First Generation	17	F	Female ticks which became completely engorged during successive feeds embracing the febrile periods of two infected guinea-pigs.
108	A16	Adult Second Generation	32	H	
187		Adult	?	J	Male ticks which fed during six-day febrile period of an infected guinea-pig.

2. *Transfer of the Virus from Larva to Nymph.*

The presence of the virus in nymphal ticks, developed from larvae which fed upon infected guinea-pigs, was proved by the demonstration of rickettsiae in stained smears and sections of their tissues and the production of infection in guinea-pigs into which they were inoculated or upon which they were fed.

Examination of Smears and Sections.

Two unfed nymphae examined by smears on the twenty-ninth and thirty-sixth days after metamorphosis contained abundant rickettsiae. Only a few rickettsiae were detected in smears of a tick examined the day after the moult, and none was detected in serial sections of a second nymph examined at the same time. Four unfed nymphae were examined by serial sections, two on the fourteenth day and two on the thirty-third day after metamorphosis. Numerous rickettsiae were seen in one of the latter ticks. No rickettsiae could be found in sections of an engorged nymph examined on the fifteenth day after metamorphosis, which was the third day after its removal from the host.

Inoculation of Guinea-Pigs.

The virus was recovered from unfed nymphae by their inoculation into guinea-pigs. Two guinea-pigs inoculated, one with three nymphs on the thirty-first day and the other with a single nymph on the thirty-sixth day after metamorphosis, became infected. The inoculation of four nymphae into a guinea-pig on the day after metamorphosis failed to induce a febrile response but conferred immunity upon the second passage animal.

The Infection of Guinea-Pigs by the Feeding of Infected Nymphal Ticks.

Nymphal ticks were fed singly and in batches of up to twenty ticks upon guinea-pigs, at intervals ranging from four to thirty-three days after metamorphosis. The average time of engorgement of the ticks was in the region of seventy-two hours.

Four of a total of twenty-four host guinea-pigs became infected. In three instances the infection was inapparent, becoming obvious on passage. The fourth animal became febrile on the twelfth day after the removal of the engorged ticks.

In all cases where fever was induced by inoculation or feeding it was proved to be Q fever by multiple transfers and immunity tests, and in many cases by transfer to mice and demonstration of rickettsiae in them.

TABLE 2.

Transfer of the Virus from Larva to Nymph.

	Feeding of Nymphae.				
Stock.	Unfed Nymphae.	Number of Host Guinea-Pigs.	Ticks per Guinea-Pig.	Day after Metamorphosis.	Result.
A	Rickettsiae detected	10	2–7 (6)	4–8	One guinea-pig infected.
	Virus isolated	1	2	33	Transmission not effected.
B	Not examined	2	2; 10 (10)	18	One guinea-pig infected.
C	Rickettsiae detected	5	1–8	24	Transmission not effected.
I	Not examined	6	1–20 (12) (1)	14	Two guinea-pigs infected.
Totals		24			Four guinea-pigs infected.

( ) Number of nymphae which fed upon the animals which were infected.

3. *Transfer of the Virus from Nymph to Adult.*

As in the case of the nymphae, the transference of the virus to the adult ticks from the preceding instar was proved by the examination of smears and sections and inoculation and feeding tests.

One hundred adult ticks were examined for the presence of virus. Fifty-four of them were dissected for the preparation of smears prior to their inoculation into separate guinea-pigs. The remainder were tested for infection by a single method; either by the examination of smears or serial sections, or by inoculation into guinea-pigs. The examinations were made at various points within the life span of the adult ticks. They were examined before feeding, at intervals during engorgement, during and after oviposition and at death. The times of these examinations ranged from one to one hundred and ten days after metamorphosis. The data relevant to the examination of the ticks are summarized in Table 3.

Fifty-nine ticks were proved to be infected. As nearly one-half of the ticks were examined only by a single method, a number of infected ticks were probably not detected. Of the total ticks examined, 44 p.c. were exposed to infection during the larval instar, the remainder during the nymphal instar. Detailed examination of members of the former group was thus made after two metamorphoses whereas the latter were examined after a single metamorphosis. The lack of any significant difference in the number of adult ticks found infected in these two groups (Table 4) suggests that the finding of only a moderate total percentage of infection was due to the failure of immature ticks to become infected, not to loss of infection during subsequent development.

Seventy-nine of the ticks were examined during or after a feed of blood, thus providing opportunity for "reactivation" of the virus. However, the percentage of infected ticks detected amongst these was not significantly higher than amongst the unfed specimens.

The rickettsiae seen in smears of dissected ticks were identical in morphology and staining reactions with those to be found in the livers and spleens of infected mice. No bodies which could be confused with the specific rickettsia under examination were seen in smears of ticks.

TABLE 3.

The Examination of One Hundred Adult Ticks for Infection with R. burneti.

Mode of Examination.	Number of Ticks Examined.	Rickettsiae seen in Ticks.	Result of Guinea-Pig Inoculation.	Number of Ticks Infected.
Smears	23	16*		16
		7°		
Smears and Guinea-Pig Inoculation	54	22*	19+	22
			2—	
			1?	
		30°	13+	13
			17—	
		2?	2+	2
Guinea-Pig Inoculation	8		2+	2
			6—	
Serial Sections	15	4*		4
		11°		
Totals	100			59

* Rickettsiae seen.
° No rickettsiae seen.
\+ Animals infected.
— Animals not infected.
? Doubtful result.

TABLE 4.

Transference of the Virus from Larvae and Nymphae to Adult Ticks.

Instar Infected.	Number of Adult Ticks Examined.	Number of Infected Ticks.	Percentage of Infected Ticks.
Larva	44	29	66%
Nymph	56	30	54%
Totals	100	59	59%

The Infection of Guinea-Pigs by the Feeding of Infected Adult Ticks.

Adult ticks were fed singly and in batches upon a series of guinea-pigs. As only 59 p.c. of the ticks were infected, and the condition of individual ticks could not be determined until after feeding, many uninfected ticks were fed upon the guinea-pigs alone or along with infected specimens.

With the exception of two guinea-pigs, upon each of which a single infected male was fed, ticks of both sexes were placed upon the animals in an endeavour to ensure impregnation and full engorgement of the female ticks. The ticks were removed from the animals after the engorgement of the females (usually 8–14 days), or after periods of attachment ranging up to twenty-six days.

Ticks which were subsequently proved to be infected were fed upon eighteen guinea-pigs, five of which became infected. The data relevant to the feeding of the infected ticks are summarized in Table 5. Uninfected ticks which also fed have been omitted from the table.

TABLE 5.

The Feeding of Infected Ticks upon Guinea-pigs.

Stock.	Host Guinea-Pig Serial Number.	Number of Infected Ticks Fed. ♂	♀	Days after Metamorphosis.	Result.
A	J564	1		5	Negative
	J565	1		5	Negative
	J567	3	2	5	Transmission effected
B	J867		1	21	Negative
	J869	2		21	Negative
	J887	1	1	21	Negative
	J892		1	29	Negative
C	J1041		2	10	Transmission effected
	J1042		1	10	Transmission effected
D	J683		1	21	Negative
	J684	3	2	21	Negative
	J722		3	48	Negative
	J755		4	18	Transmission effected
	J776		6	29	Negative
E	J1093	2	2	17	Negative
	J1095	1		17	Negative
	J1096	1	1	17	Negative
G	J1219	1	3	38	Transmission effected

Notes on the Successful Cases of Transmission.

Stock A Ticks (Infection acquired during larval instar).

Approximately five days after metamorphosis three male and two female ticks were placed upon guinea-pig J567. The ticks all became attached to the host within forty-eight hours. The two females were removed in an engorged state on the eighth and ninth days respectively. On the twelfth day the host guinea-pig became febrile and the male ticks which had been feeding intermittently were removed. The animal was sacrificed on the third day of fever. The spleen was enlarged but no other abnormality was noted. An emulsion of the liver and spleen was inoculated into a fresh guinea-pig J594 and a Q-immune animal A762. Guinea-pig J594 reacted typically with fever and was subsequently proved to be immune. The Q-immune guinea-pig A762 remained afebrile. All five ticks were found to be infected when examined after removal from the host.

Stock C Ticks (Infection acquired during larval instar).

Approximately ten days after metamorphosis two female and three male ticks were placed upon guinea-pig J1041. The male ticks were observed to feed intermittently. The two females attached themselves to the host immediately but engorged only slowly. On the fourteenth day the guinea-pig became febrile. Three days later the partially engorged females and male ticks were removed and the animal was sacrificed. An enlarged spleen was the only abnormality noted at autopsy. A fresh guinea-pig J1073, a Q-immune guinea-pig J1051 and four white mice M267–270 were inoculated with an emulsion of its liver and spleen. The test guinea-pig reacted typically with fever and was subsequently found to be immune. The immune guinea-pig remained afebrile. The mice were killed and examined seven days after inoculation when typical rickettsiae were seen in smears of their enlarged livers and spleens. It was subsequently found that only the two female ticks were infected.

Three male and two female ticks were placed on guinea-pig J1042 about the tenth day after metamorphosis. Ten days later the animal became febrile. On the third day of fever the ticks were removed and the animal was killed and passaged. Two ticks, a male and a female later shown to be infected, did not feed. The remaining female, later found infected, was partially engorged when removed, and as infection could not be demonstrated in the male ticks it alone must have been responsible for the transmission.

The infection of the host guinea-pig was proved by its passage into a fresh guinea-pig J1070, an immune animal J908 and four white mice. Guinea-pig J1070 reacted with fever and was subsequently shown to be immune. Guinea-pig J908 remained afebrile. Abundant rickettsiae were found in the tissues of the inoculated mice.

It will be noted that the ticks upon the two above-mentioned hosts fed for three days after the beginning of the fever. As infection was later demonstrated in only three of the eight ticks known to have fed, when examined immediately after removal, the interval was apparently too short for the development of infection in ticks previously uninfected.

Stock D Ticks (Infection acquired during nymphal instar).

Approximately eighteen days after metamorphosis four female and four male ticks were placed upon guinea-pig J755. Two engorged female ticks were removed after ten and thirteen days respectively. The remaining two females were removed in a partially engorged state on the seventeenth day, along with the male ticks which had fed intermittently. Five days later the animal became febrile. It was sacrificed on the third day of the fever. The spleen was enlarged. A liver-spleen emulsion was inoculated into two guinea-pigs J807 and J808, and two Q immune guinea-pigs J590 and J591. The test guinea-pigs reacted typically with fever. One was later proved to be immune. The other died from an undetermined cause before its immunity could be tested. Both of the Q immune animals J590 and J591 remained afebrile.

All four female ticks were found to be infected. The male ticks were not examined.

Stock G Ticks (Infection acquired during nymphal instar).

Approximately thirty-eight days after metamorphosis two male and three female ticks were placed upon guinea-pig J1219. The engorged females and the fed males were removed twenty-six days later. The animal was sacrificed and passaged into a fresh guinea-pig J1275 which became febrile on the eighth day. On the third day of fever it was further passaged into an immune guinea-pig J1267 which failed to react and the guinea-pig J1284 which became febrile and was later proved immune. The three females and one of the male ticks were found to be infected.

4. *Possible Transfer of the Virus from Adult to Larva.*

The progeny of sixteen female ticks exposed to infection during either their nymphal or adult instars were examined for evidence of transovarial passage of virus. In only eight of these female ticks could the virus be detected after the completion of oviposition. The other eight ticks appeared not to have been infected, and the examination of their progeny was attended with entirely negative results.

The young ticks were examined in the unfed state and at various stages during their development to maturity. A summary of the investigations made upon the progeny of the eight proved infected ticks is contained in Table 6. The progeny of one tick E32, whose infection was acquired during the nymphal instar, provided evidence of the hereditary transference of the virus. Their infectivity was suggested by the infection of a guinea-pig upon which fifteen young larvae were fed, and the finding of numerous rickettsiae in smears of a single nymph examined twenty days after metamorphosis. Other tests performed upon the same batch of ticks met with negative results. Seven nymphal and two adult ticks failed to infect guinea-pigs upon which they engorged. Likewise three unfed and four fed larvae and the two fed adult ticks failed to infect animals into which they were inoculated. These results suggest that the rickettsiae may be transferred through only a percentage of the ova to the succeeding generation.

The finding of evidence of transmission among the progeny of only one of eight proved infected ticks suggests that hereditary transmission of the virus is only an occasional occurrence. It is noteworthy that *R. ruminantium*, the causative organism of African heartwater, which *R. burneti* resembles in its apparent restriction to the alimentary canal of infected ticks, *vide infra*, has not been found to undergo hereditary transmission.

The examination of the progeny of a larger series of ticks infected with *R. burneti* is required to extend these observations and confirm the findings already described.

TABLE 6.

The Examination of the Progeny of Infected Adult Ticks.

Method of Investigation.	Days after Hatching.	Number of Ticks Tested.	Result.
Smears of Larvae	1–33	20	No rickettsiae seen
Inoculation of Larvae	1–33	192 (into 13 guinea-pigs)	All negative
Feeding of Larvae	5–70	484 (on 17 guinea-pigs)	One guinea-pig, on which 15 larvae were fed, became infected
	Days after Metamorphosis.		
Smears of Nymphae	14–20	6	Rickettsiae seen in one
Inoculation of Nymphae	9–66	37 (into 4 guinea-pigs)	All negative
Feeding of Nymphae	17–23	86 (on 9 guinea-pigs)	All negative

5. *Distribution and Multiplication of Rickettsiae within the Tick.*

A knowledge of the relations prevailing between *R. burneti* and the tissues of its arthropod host is of prime importance in determining its relationship to the other members of the Rickettsia Group. In order to study the organism and its distribution within the tick, serial sections were prepared of a number of adult and nymphal ticks, fed upon infected guinea-pigs during an earlier instar in their

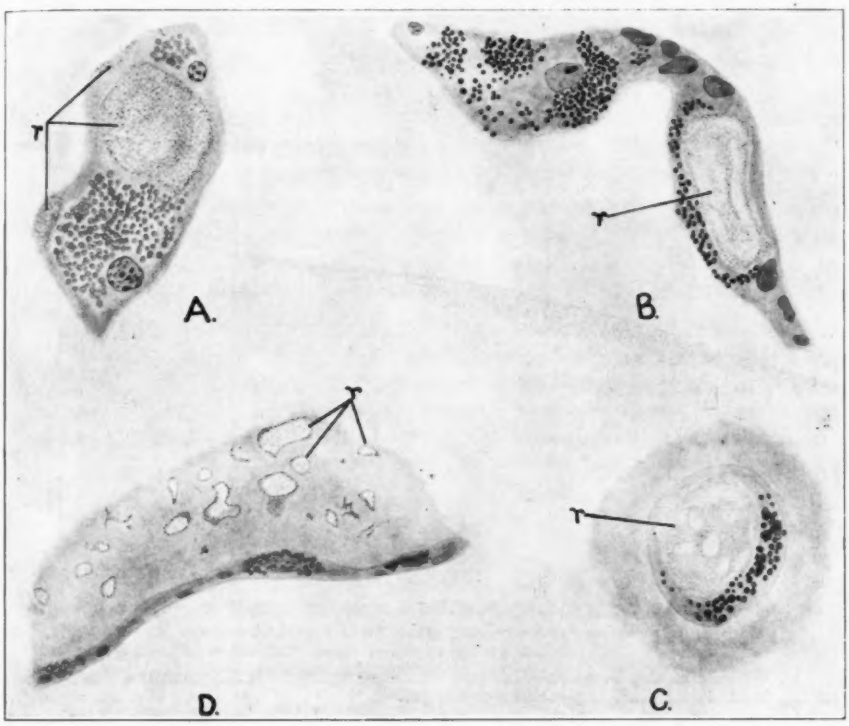

Fig. 1.

A. Two large intestinal epithelial cells containing tightly packed colonies of rickettsiae embedded within a homogeneous matrix. (r = rickettsiae.)

B. A portion of intestinal epithelium containing two hypertrophied cells, the cytoplasm of one distended with a large loosely packed colony of rickettsiae.

C. A large cell, lying free in the lumen of the midgut, containing a loosely packed colony of rickettsiae.

D. Section of a posteriorly directed caecum of the gut. Rickettsiae are absent from the non-hypertrophied cells of the epithelium which contain relatively few spherical bodies, products of the digestion and absorption of ingested blood. The lumen is devoid of free cells but contains numerous characteristic zones in which rickettsiae appear to be engaged in active multiplication.

Magnification: 450 diameters.

development. The ticks were fixed in either Zenker's or Regaud's fluid, and the sections were stained by the May-Grünwald-Giemsa method (Castaneda, 1939).

No rickettsiae were detected in a number of the ticks examined. It has already been stated that not all ticks exposed to infection appear to become infected. However, when rickettsiae were found, they were invariably present in abundance in the intestinal epithelial cells and the lumen of the gut (Figs. 1 and 2). The invasion of other organs was never observed.

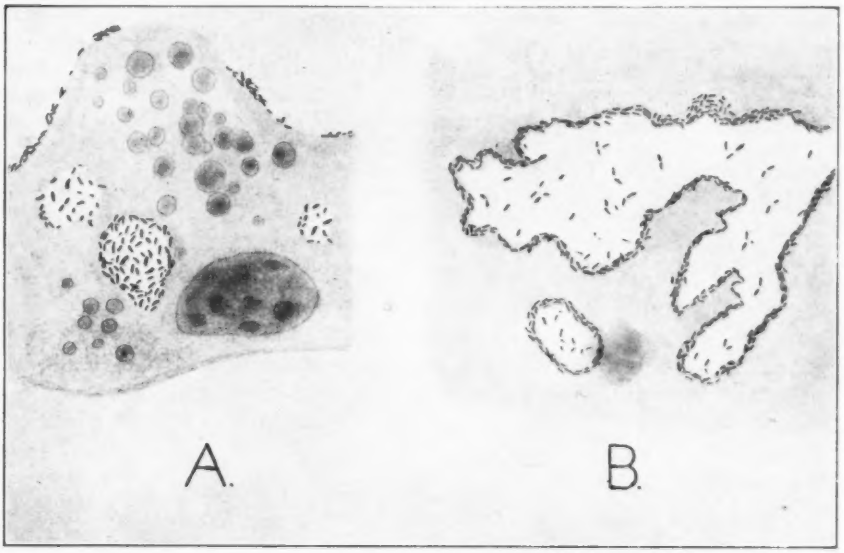

Fig. 2.

A. Intestinal epithelial cell containing small loosely packed colonies of rickettsiae, and bearing extra-cellularly several small groups of rickettsiae in the lumen of the gut.

B. Vacuolated area in the intestinal contents of an engorged tick: apparently a zone of extracellular multiplication within the lumen of the gut.

Magnification: 1,500 diameters.

Particular attention was paid to the examination of the salivary glands. Since the May-Grünwald-Giemsa stain colours the secretory globules which pack the fundus cells of the gland blue, as it does rickettsiae, it was not suitable for their examination. A number of ticks were therefore stained using the standard Castaneda methylene blue-safranin technique. Most of the rickettsiae were stained blue in these sections in which the gland cells and their contents were pink. However no rickettsiae could be detected in the glands or their ducts.

The cell infection pattern of *R. burneti* in the tick was found to be of the

typhus type as defined by Pinkerton (1936). The cytoplasm of the infected cells was often packed and greatly distended with rickettsiae which in many cases appeared to be embedded in a matrix possessing similar staining properties to themselves. This matrix, when present, was most evident in sections examined several months after staining. The blue colouration of the matrix remained fast, whereas the rickettsiae assumed a purplish hue with age. The rickettsiae were strictly confined to the cytoplasm of infected cells. The nuclei were never invaded. The histopathological findings were thus similar to those reported by Cowdry (1925) with regard to the tick *Amblyomma hebraeum* infected with *R. ruminantium*.

The appearance of the individual rickettsiae was essentially the same as when seen in infected mouse tissues. When closely packed together in heavily infected cells they appeared as minute cocci. When lying scattered in the lumen of the gut they had the appearance of small rods.

The examination of partially engorged ticks provided evidence suggestive of active extracellular multiplication of the rickettsiae within the lumen of the intestine. In the more distal portions of the caeca numerous irregularly shaped vacuolated areas of variable size were observed in the homogeneous mass of broken-down blood cells. These areas were bordered by a narrow zone of tightly-packed rickettsiae surrounding a zone of openly disposed bodies. The view that these were foci of extracellular multiplication was favoured by their early occurrence during the absorption of the digested blood and by the absence of any changes in the adjacent epithelium. In other parts of the gut diffuse masses of rickettsiae were found in association with free and degenerating cells of the hypertrophic epithelium together with the characteristic granular products of absorption, suggestive of their escape from infected cells.

Rickettsiae were never seen in the intestine anterior to the mid-gut. In the hind-gut they were found only as single bodies scattered amongst the crystalline contents of its lumen. In the examination of sections—as has already been stated with regard to the examination of smears—of infected ticks, no bodies were found which could be confused with rickettsiae. Eighteen ticks not previously exposed to infection were found to be entirely free of any rickettsia-like bodies.

6. *The Infectiousness of Tick Tissues and Tick Faeces.*

It has been reported in a previous paper (Smith, Brown and Derrick, 1939) that infected mouse tissues are capable of infecting a guinea-pig when placed upon an area of shaved skin, and that direct contact with such infective material was most probably the mode of infection in two cases of infection among the laboratory staff. Guinea-pigs into which such tissues were inoculated in 1 or 2 ml. amounts usually reacted with fever after a very short incubation period. Sometimes the temperature rose on the day after inoculation. Similar reactions, indicating a high degree of infectivity, were noted in guinea-pigs into which infected ticks were

inoculated. In order to determine whether certain cases of human infection might be attributed to contamination of the skin with squashed infected ticks, a possibility to be discussed more fully later, an attempt was made to infect guinea-pigs in this manner.

TABLE 7.

The Inoculation of Infected Adult Ticks' Faeces into Guinea-pigs.

Source of Faeces.				Number of Guinea-pigs Inoculated Primarily.	
Number of Ticks. ♂	Number of Ticks. ♀	Instar Infected.	Remarks.		Result.
3	2	Larva	Faeces collected during eighth day of ticks' engorgement. Host animal became febrile four days later.	1	Inapparent infection becoming obvious on passage.
3	2	Nymph	Faeces collected thirteen days after commencement of feeding.	1	Frank infection following one day incubation.
	4	Nymph	Faeces collected on fifth day of engorgement.	1	Inapparent infection becoming obvious on passage.
			Faeces collected on thirteenth or final day of engorgement. Host animal became febrile nine days later.	1	Frank infection following three days' incubation.
	5	Nymph	Faeces collected on fourth day of ticks' engorgement.	1	Frank infection following four days' incubation.
2	1	Nymph	Faeces collected nineteen days after commencement of feeding.	1	Frank infection following three days' incubation.
	4	Adult	Faeces collected on the tenth day after the commencement of feeding. Host guinea-pig febrile during first three days of engorgement.	1	Inapparent infection becoming obvious on passage.

An emulsion of an engorged female tick was applied to prepared areas on the flanks of six guinea-pigs. The emulsion was applied to each animal upon an area one centimetre in diameter, from which the hair had been carefully clipped to within one millimetre of the skin. Particular care was taken to choose areas free from scratches and abrasions. The tick emulsion was applied by gentle "patting" with a small porcelain pestle until the hair and skin were thoroughly moistened. All six animals became infected. In each instance the infection was inapparent in form, becoming obvious on passage (Table 8).

The discovery that rickettsiae were abundant in the mid- and hind-intestines of infected ticks foreshadowed the demonstration of the highly infectious nature of their faeces.

Samples of faeces from five batches of infected adult ticks were inoculated separately into guinea-pigs, producing infection in each instance. The faeces were

collected at intervals ranging from four to nineteen days after the commencement of the ticks' engorgement upon normal guinea-pigs. Four batches of the above-mentioned ticks had acquired their infection during the nymphal instar. Ticks of the fifth batch were infected as larvae.

Faeces were also collected from a sixth batch of adult ticks, not previously exposed to infection, during the tenth day of their engorgement upon an infected guinea-pig which was febrile during the first three days of their feeding period. These faeces also proved infective, producing an inapparent infection in the test animal. The data relevant to these examinations are summarized in Table 7.

TABLE 8.

The Application of Infected Adult Ticks' Faeces and Tissues to Abraded and Unabraded Skin.

Source of Faeces.						
Number of Ticks. ♂	Number of Ticks. ♀	Instar Infected.	Remarks.	Route.	Number of Guinea-Pigs.	Result.
	5	Nymph	Faeces collected on fourth day of ticks' engorgement.	Abraded Skin	2	Frank infections after seven and six days' incubation respectively.
2	1	Nymph	Faeces collected on the tenth day after commencement of feeding.	Abraded Skin	3	Frank infections after seven, six and six days' incubation respectively.
2	1	Nymph	Faeces collected on the nineteenth day of ticks' engorgement.	Abraded Skin	1	Failed to infect the guinea-pig.
2	1	Nymph	Faeces collected nineteen days after beginning of engorgement.	Unabraded Skin	6	Inapparent infection in all six animals, becoming obvious on passage.
Tissues of engorged female tick.			Tick infected during nymphal instar.	Unabraded Skin	6	Inapparent infection in all six animals, becoming obvious on passage.

The faeces of two batches of ticks were further investigated. Samples from one batch were applied to the members of two series of guinea-pigs upon areas of abraded and unabraded skin respectively. A sample from the other batch was applied only upon areas of abraded skin.

The abraded areas were prepared by first removing the hair from the flank with a depilatory, and making a series of light cross-hatched scratches with a needle. The faecal emulsion was applied to the prepared areas upon the two series of guinea-pigs in the manner already described.

Five or six "abraded skin" animals became infected, reacting with fever after an incubation period of seven or eight days' duration. All six "unabraded skin" animals became infected. In each instance the infection was inapparent in

form, becoming obvious on passage. A summary of the information relating to these tests is contained in Table 8.

DISCUSSION.

The study of the host-parasite relationship between *Rickettsia burneti* and its as yet only known arthropod host *Haemaphysalis humerosa* has provided further evidence of the affinities of the rickettsia with other pathogens of the genus *Rickettsia* Rocha Lima. In their arthropod host these organisms are characterized, in contrast to *Dermacentroxenus rickettsi* Wolbach, by their restriction to the alimentary canal, their failure to invade the nuclei of infected cells, and their non-transference through the ova to the succeeding generation. *R. burneti* exhibits the two first-mentioned characteristics, but there is some evidence, as yet unconfirmed, that transovarial passage of the virus may be an occasional happening.

It seems probable that *H. humerosa* infects its vertebrate hosts by the passage of virus contained in tick excrement through either broken or unbroken skin. The apparent limitation of rickettsiae to the epithelial cells and lumen of the gut, and the proved highly infectious nature of the faeces of infected ticks strongly favours this view. The relatively small percentage (approx. 25 p.c.) of successful infections induced by the feeding of ticks is also contrary to experience where direct inoculation of virus is possible, as in the instance of salivary secretions of the tick *Dermacentor andersoni* infected with *Dermacentroxenus rickettsi*. Infection by regurgitation of infective material from the mid-gut appears unlikely on similar grounds. These findings reveal a close similarity between *R. burneti* and *R. ruminantium*, the causative organism of African heartwater, in their relationships to their respective acarine hosts.

As has already been stated in a previous paper (Smith and Derrick, 1940) a history of tick bite has not been a feature of human infections. However, a certain occupational group, viz. meat-workers at the Brisbane Abattoir, amongst whom there is a relatively high incidence of infection, are exposed to contamination with the tissues of ticks infesting slaughtered animals. Tick-virus capable of penetrating unbroken skin may be the source of some cases of infection amongst these workers.

H. humerosa has once been recorded from cattle, but its incidence upon stock entering the abattoir has yet to be determined. *Boophilus microplus* the commonest tick infesting cattle in Queensland is found there in abundance, and the presence of *Haemaphysalis bispinosa* has been noted. The latter tick is found fairly commonly upon cows in the coastal areas north of Brisbane where Q fever is endemic. Roberts (1934) lists the following additional species which have been recorded from cattle and sheep in Queensland, some at least of which may occasionally be brought to the abattoir by stock. *Ixodes holocyclus, Rhipicephalus sanguineus, H. bancrofti, H. leachi, Hyalomma aegypticum, Amblyomma triguttatum*

nd *A. moreliae*. With the exception of *H. humerosa* none of these ticks has yet been adequately examined for the presence of natural infection, neither has their ability to transmit infection under experimental conditions been tested.

SUMMARY.

A study of the host-parasite relationship between *Rickettsia burneti* and the tick *Haemaphysalis humerosa* has confirmed the rickettsial nature of the pathogen, further revealed its particular affinities within the group, and suggested a possible source of certain human cases of infection.

Larval, nymphal, and adult ticks were infected with rickettsiae by feeding them upon infected guinea-pigs during the febrile period. Slightly more than half of the ticks so treated were definitely proved to have acquired infection.

Infection acquired by immature ticks was transferred through the succeeding metamorphosis or metamorphoses to the adult instar.

Infected nymphal and adult ticks were found to infect about one-quarter of the host guinea-pigs upon which they were fed.

Evidence suggestive of occasional transovarial passage of the virus was obtained, but not confirmed.

The histopathological examination of infected ticks revealed a relationship between the pathogen and its host similar to that reported by Cowdry (1925) with regard to the tick *Amblyomma hebraeum* infected with *R. ruminantium*.

In the tick host *R. burneti* appeared to be strictly confined to the intestinal epithelial cells and the lumen of the gut.

The cell infection pattern was of the typhus type. The cytoplasm of infected cells was often packed with rickettsiae but the nuclei were never invaded.

In partially engorged ticks multiplication of the rickettsiae appeared to take place both extracellularly and intracellularly.

The faeces of infected ticks were found to be highly infectious, being capable of infecting guinea-pigs when applied to either abraded or unabraded skin. This method would appear to be the natural mode of infection of the vertebrate host.

Triturated tissues of infected ticks were also found to infect guinea-pigs through unbroken skin. Some of the infections among abattoir workers may be attributed to such a mode of infection.

ACKNOWLEDGMENTS.

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REFERENCES.

Castaneda, M. R. (1939): Amer. J. Path., 15, p. 467.
Cowdry, E. V. (1925): J. exp. Med., 42, p. 253.
Jellison, W. L. and Philip, C. B. (1933): Publ. Hlth. Rep. Wash., 48, p. 1081.
Pinkerton, H. (1936): Parasitology, 28, p. 172.
Roberts, F. H. S. (1934): Animal Health Station, Yeerongpilly, Pamphlet No. 1.
Smith, D. J. W., Brown, H. E. and Derrick, E. H. (1939): Med. J. Austral., 1, p. 13.
Smith, D. J. W. and Derrick, E. H. (1940): Austral. J. exp. Biol., 18, p. 1.

STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

4. THE FAILURE TO TRANSMIT Q FEVER WITH THE CAT-FLEA *CTENOCEPHALIDES FELIS.*

by

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Although a natural tick vector of Q fever has been discovered (Smith and Derrick, 1940), many aspects of the epidemiological problem still remain obscure. This investigation was undertaken to test the ability of the common cat-flea *Ctenocephalides felis* (Bouché, 1835), which readily attacks man, to act as a vector of infection.

Ctenocephalides felis is a common parasite upon dogs—animals susceptible to Q fever (Derrick *et al.*, 1938)—cats, and calves in the country districts where Q fever is endemic. Under favourable seasonal conditions it occurs in large numbers in barns, sheds, and the farm houses themselves. Calves and dairy cows entering the Brisbane Abattoir where Q fever is classed as an occupational disease are sometimes found infested with cat-fleas. During the warmer months of the year workers engaged in removing the hides, and particularly those handling the feet, are subject to attack by fleas leaving the carcases.

TECHNIQUE.

White mice were used exclusively as the experimental animals in the feeding experiments, and with but one exception for the inoculation tests. The mice were kept separately in glass jars provided with voile covers.

Only newly emerged unfed fleas were used for experiment. The technique described by Leeson (1932) was adopted in breeding an adequate stock from a number of fleas taken from several young rabbits. It was found that ten fleas was a suitable number to feed upon one mouse provided that it was supplied with a restraining "collar". Without this "collar" the mouse rapidly ate any fleas placed in its jar. The "collar" was made from the body of a suitably sized gelatin capsule which was slipped over the mouse's head; the greater part of the capsule body being slit longitudinally into a number of strips which were bent outwards radially. A fine wire loop served to keep the "collar" in place.

The breeding and feeding experiments were conducted in an insulated room subject only to small daily variations in temperature and humidity. During the feeding of the fleas belonging to Stocks A and B the mean minimum and maximum temperatures were 74·5° and 76·5°F. with a humidity of 65–70 p.c. Stock C fleas were fed at a temperature of 80°–83°F. with a humidity ranging from 65–75 p.c.

The freedom from infection of the original fleas taken from rabbits was demonstrated by the non-occurrence of infection among the mice upon which they were placed. Their progeny were shown to be free of Q fever virus by the failure of sixty-four fleas to infect a guinea-pig into which they were inoculated. A number of fleas which were dissected and examined by smears were found to contain no rickettsiae.

Q fever infection in mice is characterized by enlargement of the spleen and the presence of micro-colonies of rickettsiae visible in stained smears of the liver or spleen. Where no rickettsiae were seen in smears of experimental mice of the first passage, their organs were inoculated into a second series of mice. As a final check these mice after examination were passaged into guinea-pigs whose temperatures were recorded daily for fourteen days after which they were tested for immunity to a strain of Q virus.

The Attempted Infection of Fleas.

Twenty-six white mice were inoculated intraperitoneally with an infective dose of guinea-pig passage virus and isolated in separate jars. These mice were not all inoculated on the same day. Ten newly-emerged fleas were placed upon each mouse. The fleas were placed upon some of the mice at the time of their inoculation and upon others after an interval of one to three days. The data relevant to the feeding of the fleas are summarized in Table 1. At the conclusion of the feeding period the mice were sacrificed and their infection confirmed by the finding of rickettsiae in stained smears of their livers or spleens.

TABLE 1.

The Attempted Infection of Ctenocephalides felis *with* Rickettsiae burneti.

		Feeding of Fleas.				Conditions of Experiment.		
Stock.	Mouse Serial Number.	Fleas Placed on Mice.	Days After Inoculation.	Days of Feeding.	* Day of Examination.	Fleas Removed from Mice.	Max. Mean Temp.	Min. Mean Temp.	Humidity.
A	274–275	20	2	18	17	9	76·5°F.	74·5°F.	65–70%
	276–279	40	1	18	16	14			
	280	10	1	17	15	10			
	282–283	20	2	16	15	18			
	284–285	20	3	15	15	8			
	287–288	20	3	11	11	19			
	289–293	50	1	13	11	39			
B	281–281a	10	1	24	24	8	76·5°F.	74·5°F.	65–70%
	286–286a	10	3	18	20	5			
C	340–345	60	0	14	11	27	83°F.	80°F.	65–70%

* Number of days after probable first feeding upon infective blood.

The Examination of Fleas.

The fleas were tested for the presence of rickettsiae at varying intervals after their first feed of infective blood, by their inoculation into mice and the examination of stained smears of their mid-guts.

Stock A. One hundred and seventeen fleas which fed upon eighteen infected mice (Table 1) were removed together, mixed, and retained over night. On the day after their removal, fifty fleas taken in batches of ten were inoculated into each of five mice. The mice were sacrificed on the seventh day when no abnormalities were noted at autopsy and no rickettsiae were seen in stained smears of either their livers or spleens. These mice were passaged separately into a second series of mice which also showed no evidence of infection. If it be assumed arbitrarily that the blood of the infected host mice became infective four days after inoculation, then the time elapsing between the first feed of infective blood and the inoculation of these fleas ranged from eleven to seventeen days. (Table 2).

Stock B. Twelve fleas which fed upon four infected mice were inoculated into a guinea-pig on the third day after their removal, which was either twenty or twenty-four days after first feeding upon infected blood. (Table 2). The guinea-pig remained afebrile for ten days after which it was killed and passaged into a second animal. The latter also failed to react with fever and was subsequently found to be non-immune.

The mice M281a and M286a which had been inoculated five days previously replaced mice M281 and M286 on the fourteenth and ninth days respectively of their fleas' feeding periods. These fleas were thus fed upon infective blood for a longer period than any others in the experiment.

Stock C. Twenty-five fleas which fed upon six infected mice were ground up together and portion of the emulsion inoculated into each of four mice. Neither these mice nor those into which they were passaged became infected. The inoculation of these fleas was made eleven days after their first feed upon infected blood. (Table 2).

TABLE 2.

The Examination of Fleas.

Stock.	Days after First Feed of Infective Blood.	Examination. of Smears.	Number of Fleas Inoculated into Mice.	Number of Mice Inoculated Primarily.	Result.
A	10	Negative (1)			
A	11 15 16 17	Negative (6)	50	5	Negative
A	21–38		38	5	Negative
B	20 24	Negative (1)	Twelve fleas inoculated into a guinea-pig.		Negative
C	11	Negative (2)	25	4	Negative

( ) Number of fleas examined.

Attempted Transmission by Feeding.

On the day following their removal from the infected host mice, sixty fleas of Stock A were placed in batches of ten upon each of six mice. These mice were sacrificed singly after intervals ranging from ten to twenty-one days (Table 3). No evidence that infection had been transmitted to the mice by the feeding of the fleas was obtained. No rickettsiae were detected in smears of the tissues of either

the host or passage series of mice, neither did the passage guinea-pigs react with fever.

The Stock A fleas used in this experiment had first fed upon infective blood from eleven to seventeen days prior to being placed upon the fresh experimental mice which they failed to infect. When each mouse was sacrificed for examination, the fleas recovered from it were inoculated into mice with uniformly negative results. These inoculations were made at intervals within a possible range of twenty-one to thirty-eight days after the fleas had first fed upon the infective blood of the original host mice.

TABLE 3.

Attempted Transmission by Feeding.

Mouse Serial Number.	Days Fleas Fed.	Fleas Placed on Mice.	Fleas Removed after Feeding Period.	Result.
M314	10	10	9	Negative
M315	13	10	–	Negative
M316	14	10	5	Negative
M317	16	10	10	Negative
M318	20	10	5	Negative
M319	21	10	9	Negative

Note. Experiment conducted at a temperature of 80°–83°C. with a humidity of 65–75 p.c.

Examination of Progeny of Fleas Exposed to Infection.

Twenty young fleas, progeny of the Stock A fleas used in the feeding transmission experiment, were ground up and portion of the emulsion was inoculated into each of three mice. Neither these nor the passage animals became infected.

DISCUSSION.

One hundred and thirty-five fleas which had been fed upon infected mice were examined for the presence of rickettsiae by dissection or inoculation tests. The examinations were made at varying intervals embracing the different periods of optimum rickettsial infection as described for the rat-flea *Xenopsylla cheopis*, in the case of the urban typhus of Malaya (Lewthwaite, Hodgkin and Savoor, 1936), and Mexican-American typhus (Dyer *et al.*, 1932). No rickettsiae were detected in these fleas, nor did a number of them produce infection in mice upon which they fed after their feeding upon infected mice.

In accord with these results was the result of the examination of a small series of cat-fleas collected from calves at the Brisbane Abattoir during December, 1937, and February, 1938. No evidence of natural infection among the fleas then examined was obtained.

In view of the failure of *Rickettsia burneti* to survive or multiply in the body of *Ctenocephalides felis*, the latter can be eliminated as a factor in the production of animal and human infections.

SUMMARY.

Rickettsiae burneti, the aetiological agent of Q fever, was found incapable of survival or multiplication within the body of the cat-flea *Ctenocephalides felis*, which thus can be eliminated as a factor in the production of animal and human infections.

ACKNOWLEDGMENTS.

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REFERENCES.

Derrick, E. H., Johnson, D. W., Smith, D. J. W. and Brown, H. E. (1938): Austral. J. exp. Biol., 16, p. 245.

Dyer, R. E., Workman, W. G., Cedar, E. T., Badger, L. F. and Rumreich, A. (1932): Publ. Hlth. Rep. Wash., 47, p. 987.

Leeson, H. S. (1932): Bull. Ent. Res., 23, p. 25.

Lewthwaite, R., Hodgkin, E. P. and Savoor, S. R. (1936): Brit. J. exp. Path., 17, p. 309.

Smith, D. J. W. and Derrick, E. H (1940): Austral. J. exp. Biol., 18, p. 1.

EXPERIMENTAL PERTUSSIS IN THE MONKEY *(MACACA MULATTA)*

by

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Reports of different workers are at variance concerning the ease with which the monkey may be infected with *H. pertussis*. Sauer and Hambrecht (1929) infected two ringtail monkeys intratracheally and one macaque intranasally, but the strength of the infecting doses is not mentioned. Sprunt *et al.* (1938) quote the same workers as having infected five cebus monkeys and three macaques by the intratracheal or intranasal routes. They give no details of dosage or of the criteria of infection.

Culotta, Harvey and Gordon (1935) used a dose of 2 to 5 c.c. of the 48–72-hour growth from three Bordet-Gengou plates (15 cm. diam.) suspended in 10 c.c. saline. By the intratracheal route they infected two of two cebus and one of sixteen rhesus monkeys. By the intranasal route one of two cebus and none of five macaques were infected. They concluded that the latter species of monkey is refractory to infection with *H. pertussis*.

Sprunt, Martin and McDearman (1938) attempted to infect erythrocebus monkeys intratracheally. They used enormous doses, the 48-hour growth of from two to twenty-four Bordet-Gengou slopes, but were successful in only two of nine attempts. One of their successes was an animal given initially the growth from twelve slopes and ten days later the same dose. When killed ten days later, twenty-five colonies were obtained on a plate inoculated from the lungs. They state "That the Bordet-Gengou bacilli failed except in two instances to multiply in the lungs of the monkeys whereas they are found in large numbers in human pertussis was attributed to the higher susceptibility of man".

Using much smaller doses than these authors, it has been found easy to infect the rhesus monkey by the intranasal and intratracheal routes. Since practically all workers have used the intratracheal route almost exclusively, while in our hands the intranasal method of inoculation has proved uniformly successful, these results seem worth recording.

METHODS.

1. *Preparation of bacterial suspensions.* The growth from a 24-hour culture of Phase I *H. pertussis* on a Bordet-Gengou slope was emulsified in nutrient broth and the bacterial content estimated by standard opacity tubes. The requisite dilutions were also made in nutrient broth, the final strength of the suspensions being 50,000,000 organisms per c.c. For intratracheal inoculations, the dose was 1 c.c. of this suspension. Intranasally, 2 c.c. were used.

2. *Method of inoculation.* The monkeys were anaesthetized with open aether. For the intratracheal inoculation the neck was extended and the needle inserted into the lumen of the trachea in the mid-line. The skin was not incised. For intranasal inoculation, the bacterial suspension was dropped into the nostrils from a Pasteur pipette.

3. *Autopsy.* Before sacrificing with chloroform, blood was withdrawn by heart puncture under aether anaesthesia. The thorax was opened aseptically, and the lungs and air-passages as high as the larynx removed. A small piece of lung tissue from each lobe was excised and the cut surface rubbed over a Bordet-Gengou plate. The trachea was divided transversely at three points and Bordet-Gengou plates inoculated from the exudate in the lumen. The root of the lungs was similarly dealt with. Specimens were selected for histological examination. In the series of monkeys infected intranasally, the autopsy included dissection and culture of the mucous membrane of the accessory nasal sinuses.

RESULTS OF INTRATRACHEAL INOCULATION.

In a preliminary trial two monkeys were inoculated. Neither developed any obvious symptoms of infection. One was killed on the seventh day after inoculation. The cut surfaces of the lungs exuded fluid, but there were no other gross macroscopic changes. A confluent growth of *H. pertussis* was obtained on plates inoculated from all lobes of the lungs, and from the tracheal exudate. The other animal was killed on the fourteenth day. The lungs, trachea, and bronchi appeared normal, and *H. Pertussis* was not isolated.

A larger experiment was then undertaken to ascertain what proportion of animals could be infected by this method and to determine the progress of the infection. Twelve monkeys were inoculated intratracheally. One was killed immediately after inoculation to determine whether sufficient organisms had reached the lower respiratory tract to be demonstrable on plating. *H. pertussis* was not recovered. Bordet-Gengou plates inoculated from various sites in the lungs contained numerous other microorganisms, presumably washed down from the site of inoculation at the upper portion of the trachea. Two animals were sacrificed twenty-four hours later with similar findings. The lungs were congested. There was an abundant growth of a mixed flora, similar to that of saliva, but *H. pertussis* was not isolated. Two more animals were killed on the fourth day. The lungs of both animals were congested. Platings of the lungs were sterile in one case; in the other a few colonies of organisms regarded as contaminants from the upper respiratory tract were found. *H. pertussis* was not isolated. Another pair were killed on the sixth day; the lungs appeared normal and were sterile. Another pair were killed on the eighth day. The lungs were congested, there was a sticky exudate in

the trachea and large bronchi, and *H. pertussis* was isolated, though not in large numbers, in cultures of the tracheal exudate and right lung in one animal, and in more profuse culture from the trachea and both lungs of the other. A final pair were killed on the eleventh day after inoculation. The lungs of both were pale and collapsed. There was abundant exudate in the trachea. A confluent growth of *H. pertussis* in pure culture was obtained from the tracheal secretion and from both lungs. These findings are summarized in Table 1.

TABLE 1.

Pathological Appearances and Bacteriological Findings in Lungs of Monkey with Experimental Pertussis.

(a) Following Intratracheal Inoculation.

Time of autopsy.	* Lung congestion.	† *H. pertussis.*	† Other organisms.
Immediately after inoculation	−	−	+++
1st day	+, +	−, −	+++, +++
4th „	+, +	−, −	+, −
6th „	−, −	−, −	−, −
7th „	+	++++	−
8th „	+, +	+, +++	−, −
11th „	−, −	++++, ++++	−, −

(b) Following Intranasal Infection.

3rd day	+, +	++++, ++	−, −
7th „	+, +, −	++++, +++, −	−, −, −
10th „	+	+++	−
13th „	+, +	−, +	−, −
17th „	−, +	+++, +++	−, −
21st „	±	−	−

* + = congestion.
± = slight congestion.
− = normal appearance.

† ++++ = confluent growth on Bordet-Gengou plates.
+++ = semi-confluent growth.
++ = numerous discrete colonies.
+ = occasional discrete colonies.
− = no growth.

The findings in the duplicate animals of this series were consistent and enable a plausible reconstruction of the course of events. The injection, under anaesthesia, of 1 c.c. of fluid into the monkey's trachea at the level of the cricoid cartilage, results in the washing into the lungs of material from the upper respiratory tract (possibly gaining access thereto from the pharynx during anaesthesia) which contains numerous bacteria. The lung responds to this insult, and inflammatory changes ensue. During this period, from the first to the fourth day following inoculation, *H. pertussis* cannot be isolated, but other organisms are present throughout the lungs, decreasing in numbers later. The lungs become normal in

appearance, and the cultures become sterile on about the sixth day. The inflammatory changes reappear, and *H. pertussis* is recoverable in pure culture, in greater numbers about the eleventh day.

RESULTS OF INTRANASAL INOCULATION.

A trial animal was inoculated intranasally and killed seven days later. *H. pertussis* in practically pure culture grew abundantly from all parts of the respiratory tract from the trachea downwards. Ten monkeys were therefore inoculated intranasally and killed at intervals of three or four days from the third day to the twenty-first day. The details are shown in Table 1. *H. pertussis* was recovered in almost pure culture from all but three animals killed on the seventh, thirteenth and twenty-first days, respectively. This method therefore certainly infected eight of eleven monkeys. It is possible that the monkeys from which *H. pertussis* was not recovered on the thirteenth and twenty-first days might have been infected at an earlier stage.

In this series, culture was also attempted from the maxillary antra and nasal mucosa. *H. pertussis* was recovered from the nose of only one monkey killed on the seventh day, and was never isolated from the antra. The findings did not support the possibility that *H. pertussis* first establishes itself in the upper air passages, and that the bronchial infection develops later.

PATHOLOGICAL CHANGES IN LUNGS.

No gross macroscopic changes were found in the lungs of any of the animals. Some appeared more deeply pink than others, but the depth of colour did not bear any relation to the degree of histological abnormality. Regarding the histological changes, the sequence of events was similar in the intratracheally and intranasally inoculated monkeys with one exception, namely, in the bronchi.

In both series, on the third and fourth days after infection a fibrinous and cellular infiltration, both alveolar and interstitial, was seen. At the end of the first week vascular congestion was prominent, whilst the cellular infiltration tended to become mostly mononuclear. The congestion was not seen in monkeys killed from the tenth day onwards, and the infiltration was then mainly interstitial. A patchy interstitial pneumonia was still evident after three weeks.

In one monkey only (killed at thirteen days) was neither histological nor bacteriological evidence of infection found.

The bronchial and bronchiolar epithelium in the intratracheally infected monkeys showed polymorphonuclear and mononuclear infiltration from the third day onwards, whilst this change was not detected in any instance in those infected intranasally. In both series the lumina of some bronchioles were filled with cells and debris, apparently deriving in part at least from desquamated bronchial epithelium.

LACK OF AGGLUTININS AND PROTECTIVE ANTIBODY.

The sera of monkeys killed at the times after inoculation shown in Table 1 were tested for agglutinins, which were not detected except in one monkey killed seven days after intranasal inoculation, from whose lungs *H. pertussis* was not isolated. This serum agglutinated *H. pertussis* (Phase I) fully at 1 : 5 and slightly at 1 : 20. The sera of two monkeys, which were inoculated intratracheally, were tested at intervals. Both were negative until three months after inoculation, when both weakly agglutinated a Phase I suspension (partially 1 : 5 and a trace to 1 : 40).

The sera were tested for protective antibody in mice by the methods described by North, Keogh, Anderson and Williams (1939). No protective antibody was detected in any sample.

DISCUSSION.

In contrast to previous workers, we have found it easy to infect the rhesus monkey with *H. pertussis* by the intranasal and intratracheal routes. In these experiments, infection was proved by recovery of the specific organism from the lungs of the animals. Had the criteria of infection consisted of the occurrence of spasmodic cough and the recovery of *H. pertussis* from the sputum, we would have had to report the same lack of success as previous workers, for none of our animals developed a cough, and consequently sputum was not available for culture in this series. *H. pertussis* was not, however, recovered from the mucous epithelium of the nose or accessory sinuses. Culotta *et al.* noted that *H. pertussis* was recoverable only from the sputa of animals with a spasmodic cough. Pertussis in the rhesus monkey is evidently usually a very mild infection, and only an occasional animal develops spasmodic cough. The association of the lack of symptoms with failure of development of agglutinins or protective antibody suggests that the rhesus monkey is capable of dealing with pertussis infection by a local cellular response in the respiratory organs. It does not seem to be a suitable animal for use in the study of experimental pertussis.

SUMMARY.

Rhesus monkeys may be readily infected with *H. pertussis* by either the intranasal or intratracheal routes. The resultant infection is mild, and spasmodic cough was not noted. Agglutinins and protective antibodies could not be detected in the sera of infected animals.

REFERENCES.

Sauer, L. W. and Hambrecht, L. (1929): Arch. Path., 8, p. 944.
Sprunt, D. H., Martin, D. S. and McDearman, S. (1938): J. exp. Med., 67, p. 309.
Culotta, Charles S., Harvey, Daniel F. and Gordon, Ernest F. (1935): J. Pediatrics, 6, p. 743.
North, E. A., Keogh, E. V., Anderson, G. and Williams, Stanley (1939): Austral. J. exp. Biol., 17, p. 275.

CRYSTALLINE HAEMOCYANIN: SOME PHYSICAL AND CHEMICAL CONSTANTS

by

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INTRODUCTION.

The study of the properties of compounds formed between metallic ions and proteins has led the author to investigate the respiratory pigment of the Australian Crayfish (*Palinurus vulgaris*). This substance is a copper-containing protein with a very large molecular weight. It is present in the blood of many invertebrate animals, but unlike haemoglobin does not occur in corpuscles. Many workers have assiduously applied to haemocyanin the methods which have been used successfully in the investigation of haemoglobin, but little information has been so obtained.

In an earlier paper (Rawlinson, 1939) it was shown that copper ions reacted with haemoglobin. Other metals investigated showed a precipitating effect, but copper solutions oxidized the haemoglobin and in the presence of oxygen denatured the protein constituent. This reactivity beween copper and iron is of added interest when one finds that copper is present in this respiratory pigment, haemocyanin, and is assumed to play a similar part to the iron of haemoglobin.

This paper describes the measurement of several physical and chemical constants of the purified crystalline pigment. In a later paper it is hoped that further constants will be recorded such as oxygen dissociation curves, molecular weight by osmotic pressure, and particularly the magnetic susceptibility of the oxidized and reduced forms of the pigment.

PREPARATION OF CRYSTALLINE HAEMOCYANIN.

Live crayfish were obtained from the local fish market, each animal being closely examined for broken limbs or ruptured membranes, because bleeding occurs very easily. The animal was tied out on a flat board with its dorsal surface uppermost and the point of attachment of the cuticle to the inferior surface of the carapace was defined by the insertion of a thin rod. A hole 3 mm. in diameter was drilled in the mid-line of the cardiac region about 4 mm. anterior to this fold of cuticle. If the outer shell was drilled carefully the underlying membrane was not split and premature loss of blood was avoided. About 20 c.c. of blood was removed with a Pasteur pipette and the remainder by inverting the animal and allowing the blood to run into a flask.

[1] This work was carried out under a grant from the National Health and Medical Research Council.

The blood clots very rapidly but potassium oxalate (0·5 gm. in 5 c.c. water for every 100 c.c. of blood) was a satisfactory anticoagulant. The collected blood was allowed to stand for about fifteen minutes with occasional mixing and was then filtered rapidly through a thin glass-wool pad to remove the small coagulum which is formed with the oxalate solution. The filtrate was then diluted with half its volume of acetate buffer solution (pH 4·6). The buffer solution contained 68 gm. hydrated sodium acetate and 28·4 c.c. glacial acetic acid per litre; this solution contains equimolecular amounts of sodium acetate and acetic acid and does not appreciably alter in pH on dilution. The blood solution was placed in the refrigerator for about seventy-five minutes. A white precipitate slowly settled out and was centrifuged off (3,000 revs. for 20 minutes). The clear blue supernatant liquid was poured into cellophane bags and dialysed against water, in the refrigerator, for about three days. The dark blue crystals of oxyhaemocyanin which formed were decanted and washed several times with distilled water until free from salts. By adding normal ammonium hydroxide solution drop by drop to a suspension of the crystals in water, solutions containing over 20 p.c. haemocyanin were prepared. Some crystals were always left undissolved in order to avoid an excess of ammonia; the solution then remained at pH 6·5.

The solution was deep blue in colour and showed one broad spectroscopic band at 5580 Angstrom units. On de-oxygenation, e.g. by evacuation, a colourless solution was formed. The haemocyanin could be recrystallized in a few minutes by mixing the solution with dilute acetate buffer solution (pH 4·6). The crystal growth could be followed under the microscope. Different experimental conditions gave different crystal forms which appeared to be all of the rhombohedral system.

Copper Content of Haemocyanin.

This was determined by three methods. Numerous preliminary experiments by ashing in a platinum crucible followed by solution in sulphuric acid and a colorimetric determination of the copper in this solution, gave values which were probably too low on account of volatilization. The values ranged from 0·071 p.c. to 0·118 p.c. and the mean was 0·093 p.c.

The two remaining methods gave concordant results. In both the pigment was digested with a mixture of sulphuric and nitric acids and the copper present was estimated either colorimetrically or electrolytically. Larger quantities of pigment were necessary for the latter.

0·50 c.c. of a 17·2 p.c. haemocyanin solution was dried on a water bath and then digested with 1·00 c.c. concentrated sulphuric acid and 0·50 c.c. concentrated nitric acid. Care was necessary to avoid violent bumping. Digestion was continued until the solution was colourless. Water was then added, the excess nitric acid boiled off, and the solution diluted to 10·0 c.c. The copper in this solution was then determined colorimetrically with sodium diethyldithiocarbamate using a control solution treated in a similar manner (Rawlinson, 1938).

The colorimetric method gave values ranging from 0·146 p.c. to 0·149 p.c. with a mean of 0·148, and by the electrolytic method a constant value of 0·148.

Isoelectric Point of Haemocyanin.

The adjustment of haemocyanin solutions to various pH values by means of suitable buffer solutions showed that in solutions less acidic than about pH 4·7, precipitation was very slow. This fact gave an indication as to the region of the

isoelectric point of the protein. A most simple and satisfactory technque was that of Freundlich and Gordon (1935). It depends on the fact that the sedimentation volume of silica particles in protein solution is greatest at the isoelectric point. The uncharged particles (at the isoelectric point) form loose aggregates which enclose a large amount of liquid, whereas the charged particles tend to glide over one another and assume a closer packing.

A silica suspension was obtained by shaking the ground powder in water and allowing it to sediment in a tube 20 cm. long for 10 minutes. This, according to Stokes' law, left particles whose diameter was about 5μ, remaining in suspension. Acetate buffer solutions were prepared, with pH values ranging from 5·3 to 4·3 and these were well mixed, in a series, with constant volumes of protein solution and silica suspension.

The sedimentation volume was found to be maximum at pH 4·60. The condensed protocol of a single experiment is given in Table 1.

TABLE 1.

Isoelectric Point.

Tube.	pH of Final Solution.	Notes.
1	4·73	Sedimentation volume small.
2	4·61*	Maximum sedimentation volume.
3	4·56	Sedimentation volume greater than tube 1.
4	4·49	Sedimentation volume small.

Specific Rotation of Haemocyanin.

The colour of oxyhaemocyanin solutions in concentrations above about 3 p.c. did not allow sufficient light to pass along a 2 dm. polarimeter tube for accurate readings to be taken.

An oxyhaemocyanin solution of 2·01 p.c. concentration was found to give a rotation of $-1·72°$ in a 2 dm. polarimeter tube when using the green line of the mercury arc. This was equivalent to a specific rotation of $-42·5°$. The solution was at pH 7·0 and 20° C.

Refractive Index of Haemocyanin.

It was found that the refractive index of an oxyhaemocyanin solution was proportional to its concentration. The concentration of the protein solution was first determined accurately by coagulation and weighing. The refractive index (R_1) of the solution was measured by means of a Pulfrich refractometer using monochromatic sodium light. A control solution (not containing the pigment) was also measured (R_2). The refraction difference (a) for 1 p.c. solution was then calculated

$$a = (R_1 - R_2) \div C$$

where C = concentration of protein in grams per 100 c.c. solution. The average value obtained gave $a = 0·00192$.

Oxygen Capacity of Haemocyanin.

Many workers have reported on methods for determining the oxygen content of bloods containing haemocyanin, but it has been difficult to find any reported oxygen determination on a solution of the pure crystalline pigment. Methods involving the destruction of the protein by heat or acids have been tried, the liberated gases being analysed for oxygen. During such processes oxygen might have been used up in the denaturation of the protein which occurred.

The most recent method is based on the fact that the cyanide radicle forms a colourless complex, with the displacement of oxygen. The use of this reagent in the Barcroft apparatus leads to grave inaccuracies. Potassium cyanide is hydrolyzed to give an alkaline solution and any buffering to a lower pH value (even above pH 7·0) causes the evolution of hydrogen cyanide. On the other hand, if the solution is made too alkaline re-absorption of gas is observed—which is probably related to an oxidative denaturation of the respiratory pigment. The pH range between which these two effects disappear is too small to allow any confidence to be placed in the results and by varying the amounts of reagents used, variable quantities of oxygen are obtained.

The double salt, potassium mercury cyanide, has been used in oxygen work. During the course of this work a sample of the double salt was crystallized. A saturated solution of potassium mercury cyanide (at 20° C.) when added to 0·25 c.c. of haemocyanin solution (18·0 p.c.) gave a steady reading, in the Barcroft apparatus, which represented a volume of 0·01065 c.c. (N.T.P.) of gas evolved. The solution however was still faintly blue but the result, which was equivalent to 23·6 c.c. oxygen per 100 grams of haemocyanin, compared favourably with the evacuation method, viz. 25·0 c.c. oxygen per 100 gm. haemocyanin. Less concentrated solutions gave unsatisfactory results owing to incomplete deoxygenation and the fact that even the use of a saturated solution is still open to this criticism made it necessary to have resort to the most fundamental method which had been used earlier to determine the validity of the chemical methods for the determination of the oxygen capacity of haemoglobin. The method involved the extraction of the gas from the oxygenated pigment by means of a vacuum and the collection of this gas for analysis.

A Toepler pump was constructed for this purpose. The main bulb of the pump was 120 c.c. capacity. The pump was connected to a drying tube of barium oxide (40 cm. × 2 cm.). This reagent, essential for the dehydration of the evolved gas, absorbed all carbon dioxide passing through it. The vessel containing the pigment solution was connected to the lower end of the drying tube. This vessel was evacuated completely by the pump and the haemocyanin solution sucked in without any air being allowed to enter. The amount of haemocyanin solution was obtained by weighing the vessel before and after the addition of pigment solution. A pycnometric density determination on a sample of the solution was performed in order to determine its volume. Pure haemocyanin solutions did not froth very much. The solution tended to become cold on account of aqueous distillation under reduced pressure and for this reason warm water was poured over the vessel from time to time. Complete deoxygenation was observed readily because the reduced pigment was colourless. The haemocyanin solutions used were equilibrated with pure oxygen and their concentrations determined refractometrically.

The volume of gas collected over mercury was measured and then an alkaline pyrogallol solution was pipetted into the burette for the absorption of oxygen. The residual gas was

measured and assumed to be nitrogen. Such nitrogen must have come from an inadvertent air leak so that the residual gas volume was corrected to an equivalent air volume. The correction for the solubility of oxygen in the solutions was important because it amounted to approximately 30 p.c. of the gas evolved.

The final results have been recorded as the volume of oxygen (at N.T.P.) combined with 100 grams of pure haemocyanin. The ratio between copper and oxygen and the minimum molecular weight have also been calculated. (See Table 2.)

TABLE 2.

Oxygen Capacity.

	1	2	3	4	5	6	Average.
Volume haemocyanin solution	26·0	28·6	35·0	51·3	41·6	61·3	
Per cent. haemocyanin in solution	15·8	18·6	21·5	22·2	20·8	21·1	
∴ Weight of haemocyanin (gm.)	4·12	5·32	7·52	11·40	8·63	12·9	
Weight of copper present (mg.)	6·07	7·88	11·25	17·00	12·9	19·3	
Corrected volume of oxygen per 100 gm. haemocyanin	25·6	26·2	22·8	24·4	24·6	26·1	25·0
Atoms of copper per molecule of oxygen	2·04	2·00	2·34	2·16	2·14	2·02	2·12

Minimum molecular weight from oxygen capacity = 89,500.

The Prosthetic Group.

Methods which were successful in identifying the prosthetic group of haemoglobin gave negative results with haemocyanin. The only hopeful result was obtained by treatment with potassium hydroxide to give a 10 p.c. solution (at 60° C. for 30 minutes). All the copper was precipitated. This method was used on whole blood containing haemocyanin as well as on the pure crystalline pigment. Earlier workers carried out similar experiments on whole blood only. The results obtained in this work showed clearly that the precipitate obtained differed in the two cases. The precipitate from whole blood was brownish green in colour, and on centrifugation gave a white layer as well as the coloured layer. That obtained from the pure crystals was dark green in colour and perfectly homogeneous. The solubility relations varied in each case. Whole blood gave a precipitate with a greater apparent solubility than the crystal product. The variations are discussed in more detail elsewhere.

Absorption of Ultra-Violet Light by Haemocyanin.

A Hilger echelon cell type of spectrophotometer was used to determine the light absorption in the ultra-violet region of the spectrum. Fig. 1 shows the results obtained under various conditions. Curves 1, 2 and 3 represent oxyhaemocyanin solutions at pH 8·80, 4·13 and 3·76 respectively. Curves 4 and 5 represent the absorption obtained after evacuation and after equilibration with carbon monoxide respectively. Complete removal of oxygen from the system was difficult

when using the open type of echelon cell, and this accounted for the incomplete disappearance of the band. Curve 6 represents the effect of potassium cyanide on the oxygenated pigment.

Carbon Monoxide Capacity of Haemocyanin.

Carbon monoxide gas was bubbled through an 18 p.c. solution of oxyhaemocyanin until the blue colour disappeared. This meant that all oxygen had been washed away from the pigment. Most earlier workers had suspected the formation of a compound between carbon monoxide and haemocyanin although not one of

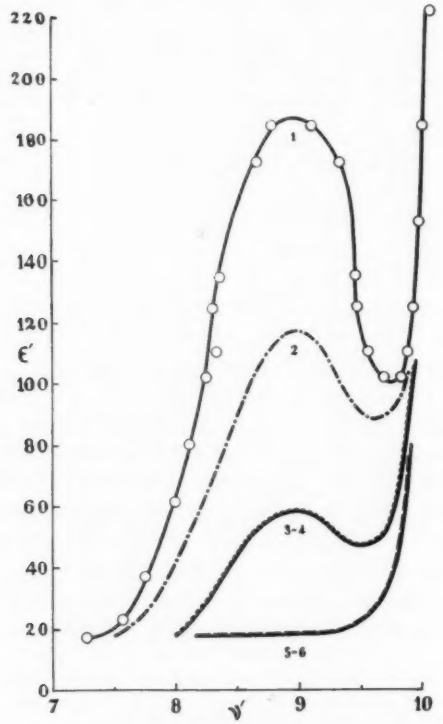

Fig. 1.

Abscissae: $\nu^1 = \nu.10^{-14}$ where ν is frequency per second.
Ordinates: $\epsilon^1 = \epsilon.10^2$

Where $\epsilon = \frac{1}{cd} \cdot \log \frac{I_0}{I}$

c = No. of grams of substance per litre of solution.
d = Depth of cell in cm.
I_0 = Intensity of incident beam.
I = Intensity of emergent beam.

hem had determined the actual carbon monoxide content of solutions. By means of a special apparatus Bland (1940) has greatly improved the iodine pentoxide method of carbon monoxide determination, and he has applied it to solutions prepared above. The results obtained have been tabulated. (Table 3).

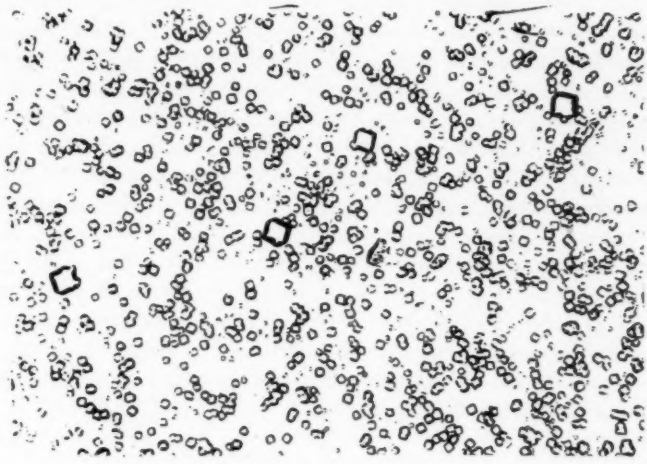

Fig. 2. Haemocyanin Crystals.

DISCUSSION.

The chromoprotein haemocyanin, which functions as a respiratory medium for many invertebrate animals, possesses unique characteristics. The remarkable ease with which the haemocyanin crystallizes from the blood of the Australian species (*Palinurus vulgaris*) under controlled conditions appears surprising when one remembers that the substance is essentially a protein. It is possible to recrystallize the pigment in a few minutes, and the actual growth may be followed by means of the microscope. (See Fig. 2.) These crystals show no tendency to dissolve in water, and are only very slowly dissolved in dilute acetic acid; in solutions more acidic than pH 4 the pigment appears to dissociate. Ammonia solutions, however, dissolve the crystals readily, and concentrated solutions may be thus prepared.

The copper content was determined on the dialysed crystals and the value obtained (0·148 p.c.) was lower than that recorded by other workers, although one report was found of 0·143 p.c. (Alsberg and Clark, 1910). These workers suggested that some of the copper dialysed away, and regarded their higher value (0·172 p.c.) obtained on an undialysed sample as more correct. Such a loss of copper by dialysis is difficult to understand because the metal must possess stable

chemical linkages to the protein in order to act as an intermediary in oxygen combination.

The isoelectric point of this haemocyanin was found to be at pH 4·6, which corresponds to values obtained by other workers in related species. The method described was found to be very satisfactory, and recommends itself for use with many other proteins.

The specific refractive difference which was found to be 0·00192 appears to be similar to previous values. Redfield (1934) quotes an average value of 0·00198, while later measurements by Roche and co-workers (1935) gave values ranging from 0·00187 to 0·00175, the value being constant for each species.

In connection with the determination of the oxygen capacity of haemocyanin, the inaccuracies of the cyanide method should be noted. The use of crystallized potassium mercury cyanide is interesting, and with modifications may be a means of avoiding the deleterious effects of potassium cyanide. The double salt is apparently ionized only to a very slight degree. The method deserves a detailed investigation in order to ascertain the exact reasons for the inaccuracies.

The evacuation method which was finally used is fundamental in principle. The corrections to be applied are important. The solutions which were evacuated contained about 20 p.c. of protein, and this must have had some effect on the solubility of oxygen. The solubility of oxygen in these haemocyanin solution was assumed to be the same as the solubility of oxygen in water. There is no real justification for this assumption, but any attempt to determine the amount of gas in physical solution would involve a tedious series of determinations at different oxygen pressures. The solubility of oxygen is probably decreased by the presence of protein, but for the present the results will remain on this assumed basis. Such solubility coefficients of oxygen in protein solutions over a range of concentrations are much needed in order to complete the oxygen capacity data. The value of 25·0 c.c. oxygen combined with 100 gm. of haemocyanin when compared with 134 c.c. oxygen for 100 gm. haemoglobin, shows clearly the inferior power of the invertebrate blood as an oxygen carrier, but this result is understandable when it is remembered that the crustacea exist under more restricted conditions than mammals. A minimum molecular weight of 89,500 was calculated from the oxygen data, but it is probable from oxygen dissociation data that about four of these units are constituted in a single molecule of the pigment. This would give an actual molecular weight of about 358,000, which is in agreement with that obtained by Svedberg from ultra-centrifugal measurements. Two copper atoms are present for every oxygen molecule, and this agrees with the values of previous workers (Guillemet and Gosselin, 1932).

The analogies between haemocyanin and haemoglobin have been over-stressed by many workers in the past as exemplified by the search for a prosthetic group. The failure of solubility tests on the product obtained from the alkaline decom-

position of the crystalline pigment, casts a doubt on much work which has been done on whole blood. Experiments which were carried out on the blood of the Australian species agreed more or less with previous reports, but when the same method was applied to samples of the crystalline pigment all tests, such as solubility in various reagents, failed to confirm the evidence previously obtained. Finally the conclusion was reached that the precipitate which formed was a basic copper sulphide contaminated or combined with protein breakdown products possessing peptizing properties analogous to those of lysalbic and protalbic acids. The whole blood of the crayfish and possibly other related species contains much protein besides haemocyanin. The use, by other workers, of such whole blood for their attempts at the isolation of an hypothetical prosthetic group offered a much better chance of such contamination of the copper compounds by peptizing colloids.

TABLE 3.

Carbon Monoxide Determination.

			Iodine liberated.
I	1 c.c.	Water	5γ
II	1 c.c.	Water saturated with carbon monoxide	35γ
III	1 c.c.	Haemocyanin solution saturated with carbon monoxide	35γ
IV		Calculated value if compound were formed	120γ

Result (I) represents the amount of iodine liberated when pure water is placed in the experimental chamber.

Ultra-violet light is absorbed by solutions of haemocyanin, and Fig. 1 illustrates the particular frequencies at which maximum absorption occurs. The band at 2780 Ångstrom units is common to most proteins and offers nothing of interest in the pigment studies. A solution of purified haemocyanin however at the neutral point shows another band at 3350 Å. If the solution is below about pH 4·0 the band at 3350 Å. decreases and finally disappears. Treatment of the pigment with potassium cyanide has a similar effect; the oxygen is known to be displaced by this process. Evacuation or equilibration with carbon monoxide affected the band; the peak of the curve was reduced but not completely destroyed because in the open type of cell used it was difficult to prevent oxygen from gaining access. All these results combine to prove that the band at 3350 Å. is caused by the oxygenation of reduced haemocyanin.

The combination of carbon monoxide with haemocyanin has been disputed for many years. In their work Dhere and Schneider (1922) suggested that no compound was formed, but the latest work of Root (1934) on the oxygen dissociation of haemocyanin in the presence of carbon monoxide led him to conclude that combination did occur. None of these workers, however, has determined quantitatively the carbon monoxide present in haemocyanin solutions. The work described in this paper gives a chemical determination of the carbon monoxide.

Table 3 shows conclusively that in oxyhaemocyanin solutions which have been decolourized by equilibration with carbon monoxide, there is no more of the latter gas present than can be accounted for by physical solution. Therefore carbon monoxide does not combine with haemocyanin to give a compound analogous to the haemoglobin compound.

SUMMARY.

This paper describes the determinations of several chemical and physical constants of crystalline haemocyanin obtained from the Australian crayfish (*Palinurus vulgaris*):

The copper content was 0·148 p.c.; the iso-electric point was at pH 4·6; the specific rotation (at 5790 Å) was — 42·5° at pH 7·0 and 20° C., and the oxygen capacity, determined by the evacuation method, was 25·0 c.c. per 100 gm. of haemocyanin.

The prosthetic group reported by other workers was considered to be a basic copper sulphide contaminated with protein breakdown products.

A diffuse spectroscopic band was observed at 5580 Å.

The ultra-violet absorption spectra consisted of a band at 2780 Å. due to the protein part of the haemocyanin molecule. A smaller band at 3350 Å. was found to be due to the oxygenation of reduced pigment.

Quantitative determinations showed that no compound of haemocyanin was formed with carbon monoxide.

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REFERENCES.

Alsberg, C. and Clark, E. (1910): J. biol. Chem., 8, p. 1.
Bland, D. E. (1940): Austral. J. exp. Biol., 18, p. 35.
Dhere, C. and Schneider, A. (1922): J. Physiol. et. Path. gen., 20, p. 34.
Freundlich, H. and Gordon, P. (1935): Trans. Farad. Soc., 31, p. 915.
Guillemet, R. and Gosselin, G. (1932): Comp. rend. Soc. Biol., III, p. 41.
Rawlinson, W. A. (1938): Austral. J. exp. Biol., 16, p. 303.
Rawlinson, W. A. (1939): *Ibid.*, 17, p. 53.
Redfield, A. (1934): Biol. Rev., 9, p. 190.
Roche, J., Roche, A., Adair, G. and Adair, M. (1935): Biochem. J., 29, p. 2576.
Root, A. (1934): J. biol. Chem., 104, p. 239.

THE ACTION OF CERTAIN SURFACE-ACTIVE AGENTS ON VIRUSES

by

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In a previous paper from this laboratory (Burnet and Lush, 1939) it was noted that there was a close correspondence between the susceptibility of viruses to a virus-inactivating agent (V.I.A.) from human nasal secretion and to sodium desoxycholate (Smith, 1939). In the hope of finding thereby some suggestions in regard to the processes involved in the inactivation of viruses by biological agents such as immune serum and V.I.A., a fairly extensive study has been made of the activity of sodium desoxycholate and two other surface active agents, sodium lauryl sulphate and saponin. All three are highly active against some viruses, much less so against others, and this paper is mainly concerned with the extent and significance of these differences in susceptibility amongst virus species.

TECHNIQUE.

The virus strains were for the most part those we have previously used in egg membrane work. The characteristics of the chorioallantoic technique make it of particular value for investigations of this sort. The results of each experiment can be read in two or sometimes three days' time and all results expressed as reduction in activity to a definite percentage of the titre of the control virus. Experience indicates that the egg membrane is very tolerant of these agents in concentrations which are fatal when inoculated intracerebrally in mice. Perhaps the greatest advantage is that of the speed with which experiments can be carried through. In animal inoculation methods it is conventional to make final readings for survival fourteen days after inoculation, and in experiments of the present type most of this period would have to elapse before the experiment could be repeated or modified. With the egg membrane technique preliminary experiments can be read at two days, and if necessary a repetition carried out with the same stock virus suspension. This is an advantage where, as in many of the present experiments, relatively small differences in virus concentrations make a big difference to the results. As a routine, virus dilutions made either in nutrient broth or saline containing 10 p.c. of normal horse serum were mixed with an equal volume of virucidal agent suitably diluted in saline and left for two hours at 36°C. before being inoculated on to the chorioallantois or by a suitable route in mice. The virus strains titrated on the chorioallantois were as follows:

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Virus strains:

(1) Herpes virus derived from the Rockefeller Institute strain H.F. and adapted to egg membrane passage (Burnet and Lush, 1939).
(2) Sabin's B virus (Burnet, Lush and Jackson, 1939).
(3) Pseudorabies virus received originally from Dr. E. Weston Hurst and slightly modified after 50 egg membrane passages (Burnet, Lush and Jackson, 1939).
(4) The egg-adapted strain of "Melbourne" influenza virus (Burnet, 1936).
(5) The virus of infectious myxomatosis as used by Lush (1939).
(6) Marchal strain of ectromelia virus.
(7) A locally isolated strain of fowl-pox virus producing typical lesions.
(8) Newcastle disease virus derived from the Victorian outbreak in 1932.
(9) Dermal vaccinia virus derived from Commonwealth Serum Laboratories' calf lymph and passaged on the chorioallantois for ten to twelve generations.

The louping ill strain was originally obtained from Moredun, and was titrated by intracerebral inoculation in mice. A psittacosis strain isolated from a human case and of high virulence for mice was titrated by the intranasal method in mice. This technique will be described in a subsequent paper from this laboratory by Rudd. The most interesting feature of the results is that with dilutions near the limit of infectivity distinct focal lesions are produced which can be counted easily and whose numbers are proportional to the amount of virus administered. Titrations of the mouse passage influenza virus strain "Melbourne" were carried out by the usual method of intranasal inoculation, and the results expressed according to the standard convention.

Chemical agents:

(1) Sodium desoxycholate (B.D.H.).
(2) Sodium lauryl sulphate, a specimen purified by Dr. I. W. Wark and kindly supplied by him.
(3) Saponin—a sample obtained from Mr. H. F. Holden, repurified by treatment with sodium oxalate and re-precipitation with alcohol.

Time, Temperature and Concentration Relationships.

Most of the detailed work with sodium desoxycholate (S.D.) and sodium lauryl sulphate (S.L.S.) has been done with egg-passage herpes virus "H.F." and with egg and mouse passage strains of influenza virus "Melbourne". With these viruses the two agents showed essentially similar behaviour, and for the most part a description will be given of the action of S.L.S. on herpes egg virus, the combination which has been most thoroughly investigated.

If time and temperature are kept constant (two hours at 36° C.) a very sharp threshold of effective concentration is found. This level depends, however, on (1) the nature of the diluting fluid and (2) the concentration of virus used. Since very dilute virus suspensions rapidly lose their activity in simple saline buffers, particularly at warm temperatures, we have always used either saline containing 10 p.c. of normal horse serum or nutrient broth (H. D. Wright's formula) as diluting fluid. As might be expected, the high protein content of the serum saline has a well-marked blocking effect on the activity of S.D. and S.L.S. It was found,

for instance, that undiluted herpes virus (suspended in broth) was much more rapidly inactivated by 0·05 p.c. S.D. than a 1 : 1,000 dilution of the same suspension in serum saline. Table 1 gives the result of an experiment in which a 1 : 100 dilution of herpes virus in saline containing various concentrations of normal horse serum was exposed to the action of suitable concentrations of S.L.S. Control titrations of higher dilutions in serum saline exposed for two hours at 36° C. indicated that the various experimental mixtures would give approximately 3,000 foci if no inactivation occurred.

TABLE 1.

Effect of Serum Protein in Diluent on Inactivating Effect of S.L.S. on Herpes Virus.

	Final dilution of S.L.S. per cent.				
Diluent.	0·025	0·01	0·005	0·0025	0·00125
100% N.H.S.	++, ++, ++	++, ++, ++	++, ++, ++		
30% N.H.S. in saline	0, 0, 0	++, ++, ++	++, ++, ++		
10% N.H.S. in saline		0, 0, 0	++, ++, ++		
1% N.H.S. in saline			0, 0, 0	3, 2, 0	++, ++, ++
Broth				0, 0, 0	++, ++, ++

++ = semiconfluent uncountable lesion, more than 100 foci.
Figures show number of foci shown on each egg membrane.
N.H.S. = normal horse serum.

The influence of virus concentration is most strikingly seen when dilutions are made in broth. The following experiment was designed after a number of preliminary experiments had shown the approximate activity to be expected with different virus concentrations.

Starting with a 1 : 10 dilution of herpes virus in broth, further closely graded dilutions: 1 : 12·5, 1 : 16·7, 1 : 25, 1 : 33 and 1 : 50, were prepared. To 0·5 c.c. of each of these dilutions an equal volume of 0·004 p.c. S.L.S. in normal saline was added, and the mixtures left two hours at 36° C. before titration on the chorioallantois. Suitable dilutions of virus ($1 : 10^4$ and $1 : 10^5$) were exposed to the same conditions with saline in place of S.L.S. and titrated. From the results of this control titration the number of foci to be expected from each of the experimental mixtures was calculated. Table 2 shows the results. The chief interest of this table is the indication that with a threshold concentration of S.L.S. there is a very marked contrast between this type of inactivation and the inactivation of viruses or bacteriophages by homologous immune serum. As Andrewes and El-

ford (1933) showed with phages, a given concentration of antiserum produces the same percentage reduction of activity irrespective of the concentration of phage. We have found this same "percentage law" to hold for several virus-antiserum reactions (Burnet, Keogh and Lush, 1937).

TABLE 2.

Influence of Virus Concentration on Inactivation by Dilute S.L.S.

Final concentration 0·002% S.L.S. with final virus dilution shown.	Foci on egg membranes.	Equivalent foci from control titration.	Approximate percentage of survivors.
Virus 1 : 20	+++ (? 500–1000)	50,000	1–2%
1 : 25	++ (200–300)	40,000	0·6%
1 : 33	60, 60, 44, 40	30,000	0·16%
1 : 50	3, 2, 1, 0	20,000	0·01%
1 : 67	0, 0, 0, 0	15,000	<0·005%
1 : 100	0, 0, 0, 0	10,000	<0·005%

We have not carried out any extensive experiments to determine the speed at which inactivation occurs under different conditions of temperature and concentration. A glance at Table 2 will indicate how difficult it would be to plan experiments in which countable numbers of foci would be obtained over the required intervals of time without using impossibly large numbers of eggs. In less ambitious experiments we have shown (1) that with relatively high concentrations (0·05 p.c.) of S.L.S. inactivation occurs within a few minutes at 0° C., (2) that with concentrations of the order 0·002 p.c. S.L.S. no appreciable inactivation is shown when mixtures made in the cold are immediately inoculated to eggs, and (3) that such mixtures show progressive inactivation up to three hours at 36° C.

Comparative Susceptibility of Different Viruses.

Owing to the influence of virus concentration on the results it is difficult to give any precise comparative figures to show the differences in susceptibility amongst viruses. A sufficiently accurate approximation may be obtained by using virus diluted (usually 1 : 100) in broth so that corresponding control titrations would give counts in the range 200–5,000. Such diluted virus is mixed with equal volumes of suitably diluted inactivating agent, and tested after two hours' contact at 36° C. Two viruses, louping ill and psittacosis, were tested in the mouse by the intracerebral and intranasal routes respectively, while Newcastle disease titrations on the egg were measured only by lethal effect on the embryo. In Fig. 1 the results are shown graphically. For each virus the concentrations of sodium desoxycholate and saponin are shown which produce complete inactivation (black), partial inactivation (shaded), and little or no inactivation (white). Similar tests on a smaller series of viruses with sodium lauryl sulphate are shown in Fig. 2.

It will be observed that the viruses can be arranged in a series which shows, with some small irregularities, an increasing resistance to all three agents. With S.D. and S.L.S. the change from one end of the series to the other is gradual, but

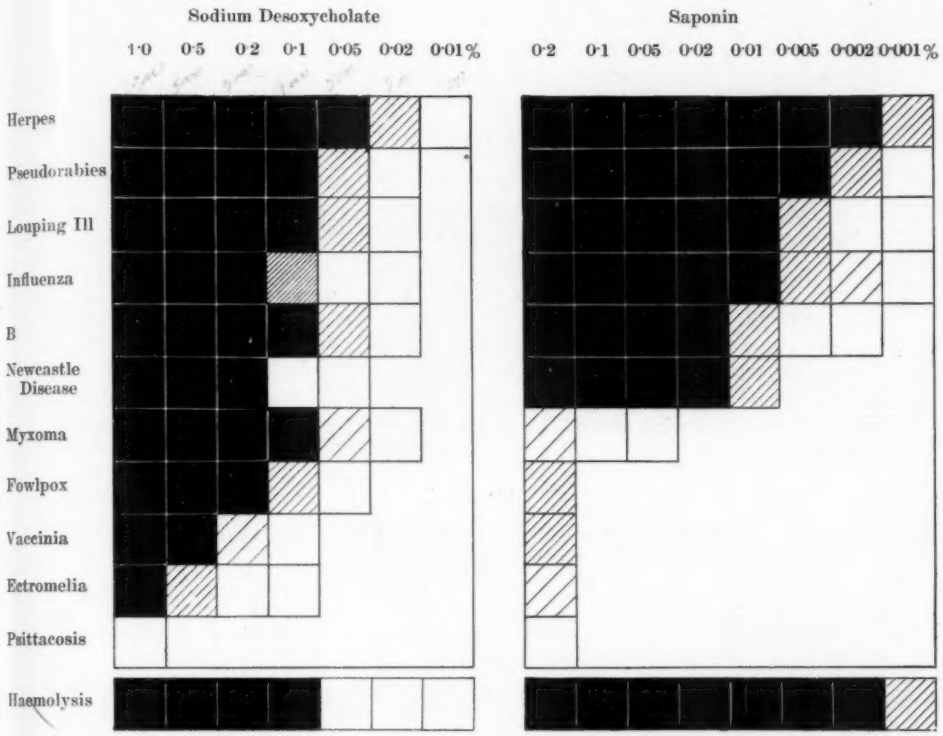

Fig. 1. The influence of sodium desoxycholate and saponin on viruses and red cells.

The effect of mixing solutions of the percentage concentrations shown with equal volumes of viruses diluted in nutrient broth is indicated in the corresponding squares. Black signifies complete inactivation, a white square no significant inactivation, and shading partial inactivation under the conditions described in the text. The haemolysis tests made with 1 p.c. suspensions of rabbit cells in saline are similarly indicated.

with saponin there is a sharp division of viruses into those highly susceptible and those almost insusceptible. Psittacosis virus is practically unaffected by the highest concentrations of any of the inactivating agents.

There is an interesting contrast in the behaviour of the substrains of "Melbourne" influenza virus which have been adapted to growth in the mouse lung

and on the egg membrane. Tables 3 and 4 show the results of comparable experiments on the two substrains. Table 3, dealing with the egg passage strain, will serve as an example of the data from which Figs. 1 and 2 were constructed. It shows a sharp threshold of activity modified in the usual way by the concentration of virus used. With mouse influenza virus, on the other hand, although there is an appreciable effect even with 0·005 p.c. S.L.S., the action increases only gradually with increasing concentration, and even with 0·1 p.c. S.L.S. fatal infections occur.

TABLE 3.

Action of S.L.S. on Egg-Passage Influenza Virus "Melbourne".

Egg-passage influenza virus.	Final concentration of S.L.S. 0·02%	0·01%	0·005%	0·0025%	0·001%	Saline.
1: 10	0, 0, 0	5, 4, 1				
1: 100			2, 2, 1			
1: 1,000			0, 0, 0	18, 18, 14, 11	++(100–200)	
1: 10,000						21, 19, 18, 17

It may be noted that S.L.S. itself in 0·1 p.c. solution produced no lung lesions, and in several such experiments we have sub-inoculated from the lesions produced with mixtures of virus with strong S.L.S. solutions and produced typical influenzal lesions.

TABLE 4.

Action of S.L.S. on Mouse Influenza Virus "Melbourne".

	Final concentration of S.L.S. 0·1%	0·05%	0·025%	0·01%	0·005%
Undiluted influenza virus	4^7, 4^7, 3	4^5, 4^5, ×			
Virus diluted 1: 100		2, 1, 1, 0, 0, 0	3, 3, 2, 2, 1, 1	3, 2, 2, 2, 2, 2	4^6, 4^6, 4^7, 3, 3, ×
Control virus 1: 10^4		4^7, 4^7, 3, 3, 3, 3			

Lung lesions are shown according to the usual convention, 4^7, for example, indicating death of the mouse seven days after inoculation with completely consolidated lungs.

Possible Significance of the Relative Resistance of Mouse Inflenza Virus.

The relatively high resistance of influenza virus shown in mouse experiments is of considerable interest, and we have made some attempts to elucidate it. As it is shown by virus diluted 1: 100 in broth we can probably eliminate the influence of associated soluble proteins in blocking the action. It seemed possible that there was a resistant fraction in the virus population used, but eight successive passages from lesions obtained with virus and 0·1 p.c. or 0·05 p.c. S.L.S. still gave virus showing the same type of behaviour as the normal strain. This result was probably only to be expected, and does not exclude the possibility that the flat curve is due to a wide range in non-inheritable variation in resistance. The action of S.L.S. on a rickettsial infection, which has been described elsewhere, suggested the possibility that the action of S.L.S. on such viruses as influenza and herpes was

not a directly lethal one, but required the co-operation of the susceptible cell. If the cells of the chorioallantois were on the average more effective in dealing with virus damaged by a chemical agent than those of the mouse lung, it would be possible to explain the difference in results without any assumption that the *in vitro* processes differed in the two instances. The most direct evidence that the "killing" of the virus is essentially a cellular phenomenon would be to show that a virus pathogenic for both mouse and chick embryo could under appropriate conditions be rendered infective for one host, but not for the other. Unfortunately in

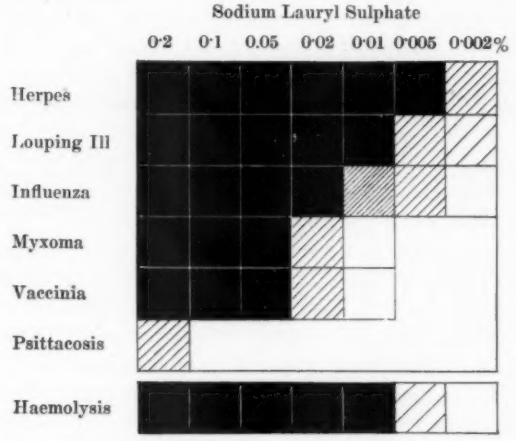

Fig. 2. The influence of sodium lauryl sulphate solutions on a smaller series of viruses. The findings are indicated in the same fashion as in Figure 1.

our experience the development of pathogenicity for the chick embryo is always associated with some loss of virulence for the mouse, and in an experiment with egg-adapted "W.S." which retains a considerable titre for the mouse, virus inactivated for the chorioallantois was also inactive for the mouse. Further experiments with partly egg-adapted strains are being made.

Action on Bacteriophages.

A representative series of bacteriophages was tested along the same lines with sodium lauryl sulphate, using a mixture of phage with 0·2 p.c. S.L.S. The phages included three dysentery phages (C.16, D.6 and D.44) of widely different character, two *Salmonella* phages, and two active against *Staphylococcus aureus*. None showed the slightest indication of any reduction in titre. Rough tests with sodium desoxycholate and saponin also failed to show any action even on the relatively delicate staphylococcal phages.

Comparison of Haemolytic and Virucidal Action.

It is a point of some interest that for each agent there is a fairly close correspondence between the concentration capable of haemolysing rabbit red cells and that effective against herpes virus. At the bottom of Figs. 1 and 2 have been added the results obtained with 1 p.c. cell suspension added to an equal volume of the dilutions shown. Since saponin is not a well-defined chemical substance we have tested another sample for its activity on herpes and vaccinia virus. It showed no action on vaccinia virus and about one-fifth of the activity of the first sample on herpes virus. Haemolytic tests showed an equivalently smaller activity.

TABLE 5.

Summary of Reactions of Viruses to Saponin, V.I.A. and Homologous Immune Serum.

Viruses arranged in order of susceptibility to surface-active agents.	Inactivation by: Saponin.	V.I.A.	Representative titre of homologous immune serum.
Herpes	+	+	0·02%
Louping ill	+	+	0·0013%
Influenza	+	+	0·001%
B.	+	+	0·06%
Pseudorabies	+	—	—
Myxomatosis	—	—	0·1%
Fowl-pox	—	—	5%
Vaccinia	—	—	1·6%
Ectromelia	—	—	1·5%
Psittacosis	—	—	100%

DISCUSSION.

These investigations have confirmed and extended the finding of Wilson Smith (1939) that viruses pathogenic for animals show a graded susceptibility to certain surface active chemicals. In Table 5 the data in Figs. 1 and 2 are used to arrange the viruses we have studied in their order of susceptibility.

There are two types of biological agents which can inactivate viruses, specific immune sera and the agent in human nasal secretion which we have referred to as V.I.A. (Burnet and Lush, 1939). In the same Table we have indicated (a) whether or not each virus is inactivated by V.I.A., and (b) representative antibody titres of active immune sera from suitable hosts. The latter figures represent the percentage of "surviving" foci obtained on the chorioallantois when undiluted serum acts on virus under our standard conditions. The sera chosen were those used in the series of investigations reported by Burnet, Keogh and Lush (1937), and they may be taken as representing the highest titre sera of each type that we had available.

It will be seen from the table that viruses may be divided into three groups:

(1) Those sensitive to V.I.A., inactivated by saponin and highly susceptible to S.D. and S.L.S., and reduced by homologous immune serum to values of the order 0·01 to 0·001 p.c.; herpes, influenza, louping ill, and B viruses. Pseudorabies, Newcastle disease and poliomyelitis viruses probably also fall in this group, but the data are incomplete. (2) Those insusceptible to V.I.A. and saponin, more resistant to S.D. and S.L.S., and with homologous immune serum, showing flat serum and concentration-survivor curves and a lower degree of inactivation, 5 to 0·1 p.c.: myxomatosis, fowl-pox, vaccinia and ectromelia viruses. (3) The final group contains only psittacosis virus, which is completely insusceptible to V.I.A., saponin, S.D. and S.L.S. in the highest concentrations tested, and is not demonstrably inactivated by immune serum. The position of psittacosis virus is of special interest. There is no doubt that complement fixing antibodies (Bedson, 1933) are regularly present in the sera of animals or human beings who have recovered from psittacosis. Yet all observers have had great difficulty in showing any inactivating effect on the virus by such antibody-containing sera. We have tested three human sera for such activity on the chorioallantois (unpublished) without finding any trace of activity. Owing to the poor foci usually produced on the chorioallantois by this virus the experiments lack precision, but the impression we received was that no reduction whatever occurred, and we can be quite definite that there was never a reduction below 25 p.c. of the control activity. Experiments made by the intranasal method in mice, to be reported in a subsequent paper, also showed no indication of any inactivation by human or guinea-pig immune sera. It may be worth noting here that our results with S.L.S., which at a final concentration of 0·1 p.c. has no action on psittacosis, but is actively bactericidal for pneumococci, may provide a useful method for the isolation of psittacosis virus from sputum containing mouse-pathogenic pneumococci.

We feel confident that the correlation in the susceptibility of viruses to immune sera, V.I.A. and surface active agents will eventually supply a clue to the nature of the process by which virus particles are rendered non-infective by the appropriate antibody. In our previous monograph on the virus-antibody reaction (Burnet, Keogh and Lush, 1937) it was concluded that, although the evidence was clear that the essential *in vitro* process was a reversible union of antibody with antigenic determinants on the surface of the virus particle, the reason why such coated virus particles failed to initiate infection in susceptible cells was still obscure. It was presumed that the antibody acted to disturb the processes by which the virus particle was capable of utilizing cell metabolites for its multiplication. We might now suggest very tentatively that inactivation within the cell is a function of some enzyme capable of disintegrating the surface structure of the virus particle in a similar fashion to that of the surface active agents we have employed. The agent in nasal secretion may perhaps be one such enzyme set free from the cells susceptible to influenza virus infection.

SUMMARY.

In addition to sodium desoxycholate, sodium lauryl sulphate and saponin show a selective inactivating effect on certain viruses.

Saponin solutions show a particularly sharp differentiation of viruses into those susceptible and those insusceptible to its action.

The action of all three agents is modified by the presence of protein in the suspending fluid and by the concentration of virus present. There are gross deviations from the "percentage law" of Elford and Andrewes.

Psittacosis virus is almost unaffected by these surface-active chemicals, and a method for its isolation from sputum containing mouse-pathogenic pneumococci by treatment with sodium lauryl sulphate is suggested.

All the bacteriophages tested are highly resistant to these agents.

Viruses can be arranged in a series of graded susceptibility which, with insignificant exceptions, is the same for sodium desoxycholate, sodium lauryl sulphate, saponin, the virus inactivating agent in human nasal secretion, and homologous immune serum.

A brief discussion of the possible significance of this correlation is given.

ACKNOWLEDGMENTS.

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REFERENCES.

Bedson, S. P. (1933): Brit. J. exp. Path., 14, p. 162.
Burnet, F. M. (1936): *Ibid.*, 17, p. 282.
Burnet, F. M., Keogh, E. V. and Lush, D. (1937): Austral. J. exp. Biol., 15, p. 227.
Burnet, F. M. and Lush, D. (1939): J. Path. Bact., 49, p. 241.
Burnet, F. M., Lush, D. and Jackson, A. V. (1939): Brit. J. exp. Path., 20, p. 377.
Burnet, F. M., Lush, D. and Jackson, A. V. (1939): Austral. J. exp. Biol., 17, p. 35.
Lush, D. (1939): *Ibid.*, 17, p. 85.
Smith, W. (1939): J. Path. Bact., 48, p. 557.

PHYSIOLOGICAL CHARACTERS AND SEROLOGICAL TYPES OF HAEMOLYTIC STREPTOCOCCI OF GROUPS B, C AND G FROM HUMAN SOURCES

by

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GENERAL INTRODUCTION.

It has been shown (Keogh and Simmons, 1940) that strains of the same serological type of Group A haemolytic streptococci have the same physiological characters. The physiological characters of Group A strains are not identical; Group A can be divided by fermentation reactions with starch, mannite and cellobiose, into sub-groups which coincide with the serological subdivisions.

We report here the examination of a collection of strains of haemolytic streptococci of human origin belonging to Groups B, C and G, and show that in these groups also there is a similar correlation between serological type and physiological characters.

PART I—GROUP B.

Several groups of workers have paid particular attention to the serological typing of Group B streptococci of bovine origin, but the human strains have been comparatively neglected. The reason is obvious. The bovine strains are of great economic and, possibly, epidemiological importance. The human Group B strains are rarely associated with serious disease in man.

During a survey of the distribution of haemolytic streptococci in Australia, a number of Group B strains were isolated from throat swabs and from excised tonsils. An attempt was made to determine whether these human strains were casual contaminants of direct bovine origin or were strains of peculiarly human habitat. If of direct bovine origin, they should be identical biochemically and serologically with the strains of *Str. agalactiae* found in Victorian dairy herds.

Previous Work on the Serological Subdivision of Group B Streptococci.

Lancefield (1934) divided the original 21 haemolytic Group B strains into three types, I, II and III, by a precipitin technique, using sera relatively rich in

type precipitin and weak in group precipitin. The divisions thus obtained did not appear to coincide with the sources of the strains, since Types I and III each included strains of human and bovine origin. Confirmatory evidence of the validity of the classification was, however, found in the results of cross-protection tests in mice, tests necessarily limited in extent by the low virulence for mice of almost all Group B strains. Stableforth (1937), comparing Lancefield's type strains with his bovine type strains, identified by slide-agglutination and agglutinin-absorption methods, concluded that Lancefield's Types II and III contained in each type representatives which were not serologically identical as judged by agglutinin-absorption.

Brown (1939) stated that 67 of his human and bovine Group B strains were "typed" by Lancefield or Plummer; that no close correlation of source, fermentative characters, and serological type was apparent; and that further study would be needed to determine whether there is any correlation.

For serological classification we have relied on slide-agglutination methods of which we have had more experience, but have made some comparative tests with the precipitin technique.

MATERIAL AND METHODS.

Human Strains. 100 human Group B strains were examined. Their origin was as follows:

Throat swabs, 25.
Excised tonsils, 34.
Superficial infections, 4.
Vaginal cultures of patients with puerperal pyrexia, 32.
Blood culture (5 fatal septicaemias), 5.

The 32 strains of vaginal origin and 4 strains from fatal cases were kindly given us by Miss Hildred Butler, who has recently discussed the clinical aspects of the cases from which they were isolated (Hill and Butler, 1940).

Bovine Strains. The serological types strains of *Str. agalactiae* were kindly supplied by Mr. D. Murnane. They include the following types, viz.: 1a, 1b, 1c, 1d, 2a, 3a, 3b, 3c, 6a, and five types isolated by Mr. D. Murnane, which, on the basis of unpublished studies, are representative of the strains present in Victorian dairy herds.

All the human strains tested gave typical double zone beta haemolysis (Brown, 1937).

Twenty-eight strains were tested for fibrinolytic action on human plasma (Tillet and Garner, 1933). None was fibrinolytic.

Preparation of Sera. Sera were prepared in rabbits with formalin-killed cocci. Satisfactory sera were prepared by weekly injections of formalized suspensions in saline, equivalent to 5 c.c., 10 c.c. and 15 c.c. of 18-hour tryptic-digest broth cultures. Sera prepared from the strains "Tracy", "Robinson", and "Abraham" were specific, requiring no absorption.

The serum prepared from strain "Hallett" cross agglutinated with strain "Gleeson" and that made from strain "Gleeson" agglutinated weakly the Lancefield strain 0·90.R. Absorption of these sera with the appropriate strain eliminated the cross reactions. All locally isolated strains provided suitable stable suspensions for slide agglutination when grown overnight in tryptic digest broth at 37°C.

Results.

Fermentation Tests.

All the locally isolated human Group B strains attacked trehalose, saccharose, mannose, maltose, galactose, glucose, laevulose, dextrin, glycerol and salicin. All strains fermented salicin overnight with two exceptions, one fermenting in eight and the other in ten days. Fermentation of glycerol was not apparent until the third to the sixth day; a few strains did not ferment until later, up to the fourteenth day. Most of the other substances were attacked overnight by the great majority of strains.

No strains fermented arabinose, raffinose, xylose, rhamnose, sorbite, mannite, adonite, erythrite, dulcite, aesculin, amygdalin or starch. Sixty-two strains attacked lactose, 38 did not.

These results agree with those of Brown (1939) who did not examine the reactions with mannose and glycerol (positive) or amygdalin, sorbite, and erythrite (negative). Brown had six human salicin-negative strains in his collection, all from a single source. We have encountered no salicin-negative strains.

Mr. E. Munch-Petersen kindly examined 42 of the strains of human origin in conjunction with many strains of *Str. agalactiae* from bovine sources. Thirty-seven lactose-positive strains satisfied the criteria for *Str. agalactiae* in respect of final pH in glucose-broth and reactions in litmus-milk, methylene blue-milk 1 : 20,000, mannite, salicin, sodium hippurate, aesculin, and growth in depth in 10 p.c. bile-salt-agar (McConkey's medium). The lactose-negative strains varied only in failing to produce acid and clot in litmus-milk, in failing to reduce methylene blue-milk, and in failing to grow in depth in 10 p.c. bile-salt-agar. The reduction of methylene blue-milk is not, however, a critical test.

Pathogenicity for Mice.

Brown noted that human strains were more frequently pathogenic for mice than bovine, and that the lactose-negative human strains were more frequently pathogenic than the lactose-positive. In tests on a few strains similar results were obtained.

Table 1.

Pathogenicity of Bovine and Human Group B Strains for Mice.

Source of strain.	Lactose.	Mortality. 3/3	2/3 or 1/3	0/3	No. of strains.
Human	—	6	1	0	7
"	+	3	4	1	8
Bovine	+	0	4	6	10

3 mice inoculated intraperitoneally with 0·5 c.c. of 18-hour broth cultures of each strain.

Serological Tests.

Eighty-six of the 100 strains fell into five agglutinative types. There was a strong but not absolute correlation between lactose fermentation and serological types (Table 2). Types "Tracy", "Robinson" and "Gleeson" accounted for 37 strains of which only three attacked lactose. Type "Abraham" included 46 strains, of which 45 fermented lactose, one from a fatal case was lactose-negative. There were five strains from fatal cases, four were lactose-negative, one was lactose-positive.

TABLE 2.

Correlation of Serological Type and Lactose Fermentation in "Human" Group B Strains.

Type.	No. of strains.	Lactose +	Lactose −
Tracy	15	0	15
Robinson	10	1	9
Gleeson	12	2	10
Abraham	46	45	1
Hallett	3	3	0
Untyped	14	11	3

2 unclassified lactose + strains were agglutinated by a serum prepared from the Lancefield Group B strain "0·90.R".

Correlation of Source of Strain and Serological Type.

None of the human Group B type sera agglutinated any of the bovine type strains. These included strains representing the types prevalent in Victorian dairy herds.

None of the human type strains was agglutinated by any of the bovine type sera. These tests were performed by Mr. D. Murnane, who also tested against all the bovine type sera the 12 human strains which were not agglutinated by any of the human type sera. One was agglutinated by, and absorbed a Victorian bovine type serum. There is therefore almost complete correlation in this collection between source of strains and serological type.

Comparison between Agglutination and Precipitin Methods of Typing.

The sera used for agglutination were prepared by a short course of immunization of rabbits. They contained no type or group-precipitins, except that the serum prepared against strain "Gleeson" contained weak group-precipitins. Incidentally this serum agglutinated the Lancefield strain 0·90.R which is rich in group-polysaccharide, and required absorption with this strain before it could be used as a type serum.

A serum prepared by prolonged immunization of a rabbit with a strain "N 954", and which precipitated formamide extracts of the homologous strain,

was tested as a precipitin serum against 10 strains each of the types "Abraham", "Tracy", "Robinson" and "Gleeson", and against 13 bovine type strains. It reacted only with the homologous "Abraham" strains. This result supports the findings of Slanetz and Naghski (1940), who concluded that both precipitin- and slide-agglutination tests gave essentially the same results, but that the agglutination test requires considerably less time, labour and serum than does the precipitin test. In addition, it is easier to prepare satisfactory agglutinating sera.

Discussion.

The results reported show that in this collection of 100 human Group B strains there was very good correlation between serological type, fermentative character and source of the strain. The types of Group B strains which were recovered from human sources in Melbourne in the past two years have not been recovered from bovine sources, and *vice versa*. The correlation between lactose fermentation and serological type is not complete, but nearly so; the lactose-negative and lactose-positive strains, with few exceptions, belong to different serological types. There is no obvious reason for the discrepancy between these findings and those of Brown (1939) who noted no such correlation. Brown's human strains were collected over a much longer period, and possibly if the present investigation had been sufficiently extended some definite overlap of bovine and human strains might have been found. But at the moment the conclusion seems justified that the Group B strains found in man are human parasites, independent of any bovine source, and that the bovine strains are not, except under exceptional circumstances, derived from human sources. If this is so, human carriers need not be considered as a possible source of infection of cattle with streptococci causing mastitis.

Part II—Group C.

The strains of streptococci originally classified by Lancefield (1933) in Group C were all of animal origin. With the wider use of the precipitin tests, it soon became clear that Group C strains were common inhabitants of the nasopharynx of man. Strains of human and animal origin can usually be distinguished by their actions on trehalose and sorbite, a test introduced for this purpose by Edwards (1932) prior to the description of precipitin grouping by Lancefield (1933). The 169 human Group C strains in this collection all attack trehalose but not sorbite. The strains were isolated at these laboratories incidentally to a survey of the distribution in Australia of the different types of Group A haemolytic streptococci. This paper reports their classification by fermentation tests and by slide-agglutination.

MATERIAL AND METHODS.

Source of Strains. 165 strains were isolated from Melbourne and Adelaide. All but two were isolated from the upper respiratory tract, mainly from throat swabs of healthy adults

and children, and from excised tonsils. One strain was isolated from urine, and one from pus from a suppurative inguinal adenitis complicating a compound fracture. Haemolytic staphylococci were also isolated from the pus. The Griffith Group C type strains "Azgazardah" (Type 7), "Neil" (Type 20) and "Angel" (Type 21) were also examined, as was the strain "F132", received from Dr. R. Lancefield and originally received by her from Dr. F. Griffith.

Identification of Strains. All strains were identified as Group C by the precipitin test, the group-polysaccharide being extracted by the formamide method of Fuller (1938).

Preparation of Agglutinating Sera. The sera were prepared in rabbits by intravenous injection of formalin-killed cocci, following the technique of Rudd *et al* (1939), except that, after the initial injections, larger doses (the centrifuged deposit from 15 c.c. of an 18-hour culture re-suspended in 1 c.c. of saline) were used than recommended by those authors for Group A. Satisfactory sera are usually obtained following three injections at weekly intervals. The sera were absorbed with Group C polysaccharide and, when necessary, with strains of heterologous types with which they show cross-agglutination.

Method of Agglutination. Agglutination tests were performed by Griffith's slide technique. Each serum was used in the greatest dilution in which it gave a ++++ reaction as figured by Pauli and Coburn (1937), usually 1: 20 or 1: 40.

The majority of Group C strains grow flocculently in tryptic-digest broth, and the untreated deposits provide extremely unstable suspensions. This difficulty was overcome by the use of the method of V. D. Allison (unpublished), the principle of which is incubation of the suspension with pancreatic extract.

Media for fermentation tests. The carbohydrates, except starch and aesculin, were used in 1·0 p.c. concentration in the following basic medium, adjusted to pH 7·1, and sterilized by Seitz filtration: Proteose peptone (Difco), 10 gm.; sodium chloride, 5 gm.; brom cresol purple (1·6 p.c. solution in 95 p.c. ethyl alcohol), 1·0 c.c.; water to 1 litre.

Starch was used according to the following formula: Proteose peptone (Difco), 10 gm.; sodium chloride, 5 gm.; soluble starch (B.D.H.), 1 gm.; Andrade's indicator, 10 c.c. Water to 1 litre. Warm to dissolve, tube, and autoclave 15 minutes at 115°C.

There was some trouble in obtaining satisfactory results with aesculin broth. The medium used was essentially that of Harrison and Vanderleck (1909), omitting the bile salt and adjusting the pH to 7·2. Batches varied slightly, presumably owing to variation in the time and temperature of sterilization in the autoclave. More satisfactory results were obtained when the medium was sterilized by Seitz filtration. Some strains did not appear to grow well and the medium was further modified, reducing the amount of iron citrate, to the following formula: Aesculin, 0·05 gm.; ferric citrate (scale), 0·025 gm.; tryptic digest broth (Ward and Rudd, 1938), 100 c.c. or peptone-water base 100 c.c. Sterilize by Seitz filtration and tube.

The fermentation tests were observed for 14 days. Fermentation of raffinose was always complete in 24 hours; fermentation of aesculin, amydalin and lactose was usually complete in 24 hours, occasionally delayed to the second or third day, and only very exceptionally was there a longer delay.

RESULTS.

All strains attacked the following substances: salicin, saccharose, maltose, galactose, mannose, laevulose, glucose, dextrin, starch, glycerol and trehalose. (With trehalose there was one exception. Of two colonial variants picked from the original plating of a throat swab, one attacked trehalose, the other did not. The two strains were serologically identical and agreed in all the other fermentation reactions.)

None of the following substances was attacked: sorbite, mannite, xylose, arabinose, adonite, erythrite, inulin, dulcite, rhamnose and inosite, with the exception of that one strain which fermented inulin.

The results with aesculin, amygdalin, raffinose and lactose varied with different strains. All these substances were fermented by the single strain which fermented inulin. The 169 strains were divisible into seven sub-groups by their actions on aesculin, amygdalin, raffinose and lactose. Using these groupings as an indication of probable serological differences between strains, the production of agglutinating sera from selected strains was proceeded with, and the entire collection of strains was divided into eight serological types. The fermentative and serological groupings were not coincident, for strains which were serologically homologous, as shown by reciprocal absorption, varied in respect of fermentation of lactose. However, strains serologically homologous gave identical fermentation reactions with aesculin, amygdalin and raffinose. By their action on these three substances, strains were divisible into four fermentative groups, and two of these groups—(a) aesculin-, amygdalin- and raffinose-negative and (b) aesculin-positive, amygdalin- and raffinose-negative—were divisible into sub-groups serologically. The results are shown in Table 3.

TABLE 3.

Correlation of Serological Type and Biochemical Characteristics in Group C Haemolytic Streptococci.

Serological Type.	No. of Strains.	Aesculin.	Amygdalin.	Raffinose.	Lactose.	
					—	+
"Radford" (Lancefield, F132)	21	—	—	—	21	
"Angel" (Griffith, Type 21)	1				.	1
"Doyle"	68				17	51
"Neil" (Griffith, Type 20)	18	+	—	—	7	11
"Azgazardah" (Griffith, Type 7)	1				1	.
"King"	1				1	.
"Petersen"	29	+	+	—	12	17
"Ryan"	30	+	+	+	.	30

All the Australian strains which did not ferment any of the three key carbohydrates belong to the same serological type. "Radford" (Lancefield F132) is the type strain. The English strain "Angel", which is the Griffith Type 21 strain, is a different serological type, of which there are no representatives in the collection of Australian strains. The strains which are aesculin+, amygdalin—, raffinose—include representatives of four serological types. Type "Doyle" has the greatest number of representatives, namely, 68, but the majority of these strains were isolated in one institution, some of them being repeated isolations from a few carriers.

There are no strains corresponding to Griffith Type 7 in this collection. The strain "King", which is serologically unique, was isolated from pus, and is the only one in this collection from such a source. The local strains, which are aesculin−, amygdalin+, raffinose−, were homologous serologically, as were those attacking all three carbohydrates.

The activity of the strains towards lactose roughly parallels their activity towards aesculin, amygdalin and raffinose. Of 22 strains negative with these three substances, 21 fail to ferment lactose: while the 30 strains which attack all three also ferment lactose. Of 117 strains which attack aesculin but not raffinose, 79 ferment lactose and 38 do not.

DISCUSSION.

The haemolytic streptococci of Group C are merely opportunist pathogens rarely giving rise to serious infections in man. The findings reported are therefore mainly of theoretical interest. They reinforce those reported for the pathogenic streptococci of Group A (Keogh and Simmons, 1940), in which group there is a similar correspondence of serological and physiological characters. The preliminary classification by fermentation reactions is of great assistance in the selection of strains for preparation of sera in the early stages of serological typing of a large number of strains. Further, the fact that strains of the same serological type behave in the same way biochemically makes it certain that the divisions revealed by the slide-agglutination technique are fundamentally significant.

PART III—GROUP G.

The identification of Group G strains of beta haemolytic streptococci was reported by Lancefield and Hare (1935). Their strains were isolated from the human vagina. Bliss (1937) showed that whereas the majority of strains of "minute" beta haemolytic streptococci belong to Group F, five of 55 such strains examined by her belonged to Group G. It can be inferred from the context that these five strains fermented lactose, salicin and trehalose, although Bliss does not make the direct statement.

Although Group G strains are commonly encountered in the human throat and and vagina, they are rarely the cause of serious disease in man. Macdonald (1939) reported two fatal infections from Melbourne, and Bryce and Avery have recently encountered a similar fatal endocarditis due to a Group G strain (personal communication).

In this section we report the physiological and serological characters of 78 Group G strains isolated in Australia, of which eight are "minute" strains.

METHODS.

The methods were those described in the previous sections of this paper. Suspensions of the large colony strains were usually unstable but responded to digestion with pancreatic extract. Fermentation tests were examined for 14 days.

Results.

Fermentation of Carbohydrates.

All strains attacked glucose, laevulose, galactose, maltose, mannose, saccharose, trehalose and dextrin. None attacked arabinose, rhamnose, xylose, mannite, sorbite, adonite, erythrite, dulcite or inulin.

The reactions in lactose, salicin and aesculin varied with different strains of the same serological type among the large colony strains. The eight "minute" strains attacked all three substances, but had more strains been examined we would possibly have encountered exceptions to these findings.

The large-colony strains attacked starch and glycerol, the "minute" strains did not. The large-colony strains did not attack raffinose or amygdalin, the "minute" strains did.

The findings are summarized in Table 4.

Table 4.

Correlation of Serological Type, Colonial Size, and Biochemical Characteristics in Group G Haemolytic Streptococci.

Large colony type.	No. of Strains.	Starch.	Glycerol.	Raffinose.	Amygdalin.
Type "Cole"	62	+	+	−	−
Type "Auburn"	7	+	+	−	−
Type "Harrison"	1	+	+	−	−
Minute colony type.					
Type "Cuthell"	4	−	−	+	+
Untyped	4	−	−	+	+

Exceptions:
One minute type "Cuthell" fermented glycerol at 8 days.
One large type "Cole" fermented raffinose at 1 day.
Two large type "Cole" fermented amygdalin at 1 day and 5 days, respectively.

Serological Differentiation.

The large-colony strains could be divided into three serological types, 62 of 70 belonging to a single type "Cole". Strains of different types had the same fermentation reactions.

Four of the "minute" strains were agglutinated by a serum prepared from one of them, and four were not. There is evidence that more than one serological type is present, but the fermentation reactions of all strains were identical.

The strains from the three fatal cases referred to were all of type "Cole", which has evidently been the predominant strain in Melbourne for many years, as we have strains of this type isolated from 1929 to the present date.

Discussion.

There is a clear-cut distinction between the "minute" G strains and other members of the group. This was noted by Sherman (1937), who found that all the "minute" G strains in his possession fermented raffinose, curdled milk and were devoid of strong fibrinolytic action. We would add to these characteristics fermentation of amygdalin and failure to attack starch and glycerol. The physiological characters of the large-colony members of the group which we have studied are uniform, irrespective of serological type, except that there is some variability in respect of fermentation of lactose, salicin and aesculin. Fermentation of lactose appears to be of little significance in streptococci of human origin, as strains of the same serological type vary in their capacity to attack it.

SUMMARY.

Part I—Group B.

100 Group B strains of human origin have been tested for fermentation of a large number of carbohydrates, and have been serologically "typed".

There is close but not absolute correlation between ability to ferment lactose and serological type.

Human Group B strains and bovine Group B strains belong to serologically distinct types, but one untyped human strain was encountered which was agglutinated by a Victorian bovine type serum.

Part II—Group C.

165 Group C strains of human origin have been tested for fermentation of a large range of carbohydrates, and have been serologically "typed".

Group C strains can be classified by the differential reactions as regards fermentation of aesculin, amygdalin, and raffinose.

The resultant grouping is consistent with that established by serological methods, but is less complete.

Part III—Group G.

The physiological and serological characters of 78 strains of Group G haemolytic streptococci from human sources are described.

Strains giving rise to large and minute colonies respectively differ in fermentation of starch, glycerol, raffinose and amygdalin.

There are several serological types of both "large" and "minute" strains which show no overlap.

REFERENCES.

Bliss, Eleanor A. (1937): J. Bact., 33, p. 625.
Brown, J. H. (1937): J. Bact., 34, p. 35.
Brown, J. H. (1939): J. Bact., 37, p. 133.
Edwards, P. R. (1932): J. Bact., 23, p. 259.
Fuller, A. T. (1938): Brit. J. exp. Path., 19, p. 130.
Harrison, F. C. and Vanderleek, J. (1909): Cent. Bakt., I, Abt., Orig. 51, p. 607.
Hill, A. M. and Butler, Hildred M. (1940): Med. J. Austral., 1, p. 293.
Keogh, E. V. and Simmons, R. T. (1940): J. Path. Bact. 50, p. 137.
Lancefield, R. C. (1933): J. exp. Med., 57, p. 571.
Lancefield, Rebecca C. (1934): J. exp. Med., 59, p. 441.
Lancefield, Rebecca C. and Hare, R. (1935): J. exp. Med., 61, p. 335.
Macdonald, Ian (1939): Med. J. Austral., 11, p. 471.
Pauli, R. H. and Coburn, A. F. (1937): J. exp. Med., 65, p. 595.
Rudd, G. V., White, Colin and Ward, Hugh K. (1939): Austral. J. exp. Biol., 17, p. 25.
Sherman, J. M. (1937): Bac. Rev., 1, p. 3.
Slanetz, L. W. and Naghski, J. (1940): J. infect. Dis., 66, p. 80.
Stableforth, A. W. (1937): J. Path. Bact., 45, p. 263.
Tillett, W. S. and Garner, R. L. (1933): J. exp. Med., 58, p. 485.
Ward, Hugh K. and Rudd, G. V. (1938): Austral. J. exp. Biol., 16, p. 181.

1962

THE INFLUENCE OF ANTERIOR PITUITARY EXTRACTS ON THE DETOXICATION MECHANISMS OF THE DOG[1]

by

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From the experiments of Brakefield (1927) it appears that, in man, benzoic acid is excreted only as hippuric acid. This is in marked contrast to what is found in some animals (for example, the dog), in which, in addition to hippuric acid, portion of the administered benzoic acid is excreted as the glycuronate. From experiments on dogs, Quick (1932) has shown that only one-quarter of the administered benzoic acid is excreted as hippuric, the remaining three-quarters being excreted as benzoyl-glycuronic acid. This worker (1926), studying the excretion of conjugated glycuronic acids, showed that pancreatectomy did not affect the excretion of benzoic acid as glycuronic acid. In such experiments, however, there was a marked decrease in urinary glucose with a corresponding fall in the D : N ratio, whilst the D + glycuronic-acid : N ratio corresponded quite closely to the D : N ratio of the pancreatectomized animal prior to the administration of benzoic acid.

These results led to the conclusion that in the fasting depancreatized animal both glucose and glycuronic acid have the same precursor, namely, tissue protein.

In view of the effects produced on carbohydrate metabolism by anterior pituitary extracts, it was thought worth while to determine whether such extracts caused any alteration in the elimination of benzoic acid in the dog.

This paper describes a curious phenomenon which was encountered in two dogs which had received numerous injections of a crude saline extract prepared from the anterior pituitary glands of oxen. The different types of extracts used in the experiments on dog "T" were prepared from two batches of glands, A.P.1. and A.P.2., the only difference between them being that the former were removed from the animals about six to eight hours after death, whilst the latter were obtained within twenty minutes of slaughtering. In all instances the anterior lobes were dissected from the frozen gland and stored in the refrigerator until required. Details of the extracts used and their preparation are given below.

[1] This work was carried out under a grant from the National Health and Medical Research Council.

Extract A: Prepared from A.P.1. which had been dried in acetone, powdered and stored in vacuo at 0°C. One gm. of this powder was equivalent to four gm. of the fresh gland.

Extract B: Prepared from A.P.2. which had been dried and treated similarly to A.P.1.

Extract C: Prepared from the fresh untreated A.P.2.

Preparation of Extracts:

The whole glands or the acetone-dried powder were ground up in a mortar with 2 ml. of 0·9 p.c. solution of sodium chloride to each gm. of fresh gland or its equivalent in the case of the dried powder. The saline used in preparation of the extracts for dog "T" contained merthiolate (Ely Lilly & Co.) in a concentration of 1/1000. No merthiolate was used in the extracts administered to dog "W". The mortar was placed in a freezing mixture and its contents allowed to extract with frequent stirring for one hour before centrifugation. To avoid any possible deterioration due to oxidation, etc., only small quantities of extract sufficient for two days' use were prepared at a time and kept at 0°C.

All the injections were given intraperitoneally and the dogs were placed in metabolism cages when urine specimens were required. Throughout the course of the injections the animals were given a liberal supply of fresh beef together with some cooked meat and bones. Sugar analyses were carried out by the method of Hagedorn and Jensen, the blood being collected from the marginal ear vein. The treatment of dog "T" is summarized in Table 1.

TABLE 1.

Days.	Type of Extract.	Vol. injected ml.	Day.	Fasting Blood Sugar mg. p.c.
0	—	—	0	104
1st–13th	Ext. A	10	5th	113
			7th	127
			9th	134
			11th	88
14th–16th	Ext. B	20	15th	97
17th–23rd	Ext. C	10	*19th	180
			*20th	204
			*21st	280
			22nd	113
24th–26th	do.	15	24th	97
27th–28th	do.	23	28th	106
29th–34th	do.	25	34th	90
35th–47th	do.	30	47th	104
48th–54th	do.	20	54th	99

* Glycosuria present.

Numerous blood samples for sugar analysis were taken, but only those which are relevant to the sodium benzoate experiments are included. Ketosis was not detected during the course of the experiments, although a slight albuminuria was constantly present. This apparently is not of great significance, since it is frequently found in healthy dogs. From Table 1 it can be seen that there was no definite effect of the extracts upon the blood sugar until the 19th day after the commencement of the injections, at which time the fresh gland extract (Ext. C) was being administered. A marked elevation of the blood sugar occurred on the 19th day, and the animal developed a glycosuria which reached a maximum on the 21st

day, but disappeared on the following day. From this time onward, however, despite continued injections and increasing dosage of the extract, the urine contained no reducing substances. Furthermore the fasting blood sugar remained within normal limits.

During the period between the 19th and 22nd days dog "T" was developing symptoms of diabetes similar to those described by Young (1939). According to this worker, if at this stage the dosage of the extract is increased, the blood sugar will rise further, glycosuria and polyuria will become more marked, and symptoms characteristic of permanent experimental diabetes will arise.

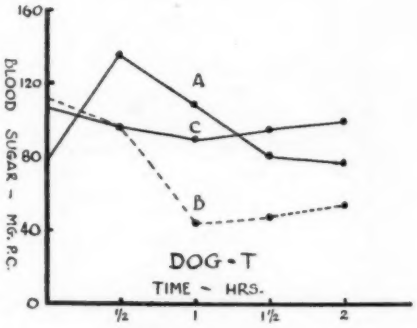

Fig. 1. (Dog "T").

Curve A. Glucose tolerance curve following the oral administration of 1 gm. of glucose per kg. of body weight. No treatment with anterior pituitary extracts.

Curve B. Blood glucose following the subcutaneous injection of 10 units of insulin. No treatment with anterior pituitary extracts.

Curve C. Blood glucose response to 10 units of insulin following treatment with anterior pituitary extracts for 35 days.

As can be seen from Table 1, this did not occur in this animal. Despite the presence of a normal blood sugar, it was interesting to note that a resistance to the hypoglycaemic action of insulin was present. This is shown in Fig. 1.

TABLE 2.

Days.	Type of Extract.	Vol. injected ml.	Day.	Fasting Blood Sugar mg. p.c.
0	—	—	0	97
1st–3rd	Ext. C	20	1st	97
			2nd	124
			3rd	125
4th–7th	do.	30	4th	146
			6th	104
			7th	124
8th–23rd	do.	40	19th	117
			20th	184
			21st	90
			22nd	93
			23rd	113

Table 2 illustrates the treatment of dog "W", which at no time exhibited any significant rise in the blood sugar, nor was there any glycosuria, polyuria or ketonuria.

The investigations on the responses of this animal to insulin and the administration of glucose were rather more thorough than was the case with the dog "T", and those results which are pertinent are illustrated in Fig. 2.

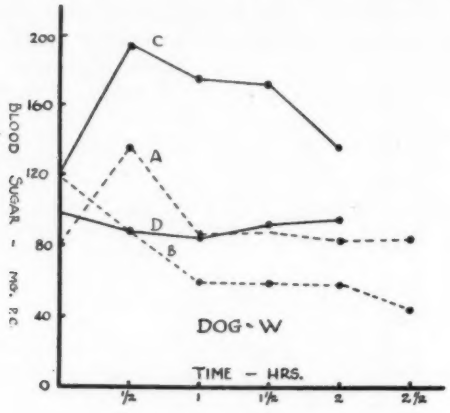

Fig. 2. (Dog "W").

Curve A. Glucose tolerance curve following the oral administration of 1 gm. of glucose per kg. of body weight. No treatment with anterior pituitary extracts.

Curve B. Blood glucose following the subcutaneous injection of 10 units of insulin. No treatment with extracts.

Curve C Glucose tolerance curve following the oral administration of 1 gm. of glucose per kg. after treatment with anterior pituitary extracts for 17 days.

Curve D. Blood glucose response to 10 units of insulin following treatment with anterior pituitary extracts for 20 days.

From a consideration of Fig. 2, wherein the curves A and C represent the response of the animal to 1 gm. of glucose per kg. before and after treatment with the anterior pituitary extracts, it is seen that on the 17th day there is a marked impairment in glucose tolerance. A 12-hour sample of urine obtained at the conclusion of this test showed traces of glucose. In addition, as shown by curves B and D, there is a well-marked resistance to the hypoglycaemic action of insulin. Treatment with sodium benzoate was commenced on the 23rd day, and the details concerning its elimination in the urine are given in the following section.

Experiments with Sodium Benzoate.

Sodium benzoate was given orally, subcutaneously or intraperitoneally, and the animals were placed in the metabolism cages for the collection of urine. In the case of subcutaneous and intraperitoneal injections, the solutions, prior to injection, were neutralized to litmus with a few drops of concentrated HCl.

Methods for the Estimation of Benzoyl Glycuronic Acid and Glucose.

If necessary, the urine was made slightly acid by the addition of 10 p.c. acetic acid and a sufficient volume of a saturated solution of neutral lead acetate added to precipitate phosphates, etc., care being taken to avoid any excess of this reagent. The mixture was then centrifuged, the supernatant fluid neutralized with 10 p.c. NaOH, and aliquots withdrawn for estimation of the reducing substances by the method of Bertrand. The rotatory power of the solution was determined before neutralization, using a 2 dm. tube and Hg light of a wave length of 5460 Å.

5 ml. of a 10 p.c. suspension of well washed baker's yeast was added to an equal volume of the neutralized centrifugate and the mixture placed in a bath at 37°C. for 1 hour. The solution was then centrifuged and the reducing power of the supernatant fluid determined as before. The results of these experiments are set out in Table 3.

TABLE 3.

Dog.	Sodium benzoate. Dose. gm.	Sodium benzoate. Vol. ml.	Method of administration.	Vol. of 24 hr. specimen. ml.	Rotation 20°C. Hg 5460 Å.	Reducing substance* gm. p.c. Before fermentation.	Reducing substance* gm. p.c. After fermentation.
"T"	3	10	Oral	620	+ 0·34°	0·66	—
(Treated as shown	10	40	„	410	+ 0·32°	0·55	—
in Table 1)	3	20	„	—	+ 0·50°	0·92	0·48
	10	50	Subcut.	705	+ 0·26°	0·65	0·29
"W"	7	20	Intrap.	1,700	+ 0·60°	1·40	0·54
(Treated as shown	7	20	„	900	+ 0·69°	1·12	0·65
in Table 2)	7	20	„	1,100	+ 0·75°	1·14	0·50
	Normal Control Animals. (No A.P. extract given.)						
1	5	50	Oral	470	− 0·40°	0·37	—
	5	50	Intrap.	825	− 0·04°	0·74	0·73
2	10	50	Oral	540	− 0·17°	1·4	1·4
3	7	60	Subcut.	215	− 0·55°	1·89	—
4	7	60	„	500	+ 0·1°	1·35	—
5	7	50	„	345	—	1·2	1·2
6	5	50	Intrap.	700	+ 0·09°	0·63	0·66
7	5	50	„	505	− 0·35°	0·87	0·83
8	10	50	„	500	+ 0·09°	0·65	0·61
9	10	50	„	415	− 0·39°	—	—

* The percentage of reducing substance is in all cases calculated as glucose.

It should be noted here that benzoyl glycuronic acid has a negative rotation, and thus, as most of the administered benzoic acid is conjugated with glycuronic acid and excreted as the glycuronate, it would be expected that the urine obtained from a dog after treatment with sodium benzoate would exhibit a negative rotation. Generally speaking this is the case in the normal animal, but in the experiments conducted on dogs "T" and "W" a markedly positive reaction was invariably found. This fact, in conjunction with the decrease in reducing power following fermentation, indicates the presence of a dextro-rotatory reducing, fermentable sugar, which is believed to be glucose.[2] In the case of these animals, the figures given in Table 3 show that about one-half of the total reducing power of the urine was due to glucose.

[2] A phenylosazone was isolated from the urine having the crystalline structure characteristic of glucosazone. Melting point and mixed melting point determinations confirmed the presence of glucose.

In the case of the normal untreated animals, Nos. 1 to 9 in Table 3, entirely different results are observed. After benzoate the urine exhibited a negative rotation, except in the case of three animals, Nos. 4, 6 and 8, where a slight positive rotation was observed. In no case, however, could any fermentable substance be detected in the urines from these animals. An explanation of this positive rotation may be that in these urines a certain amount of decomposition had occurred with the splitting-off of dextro-rotatory glycuronic acid from benzoyl glycuronate.

As a possible explanation of the above results it was at first thought that the animals "T" and "W" might have had lowered renal thresholds for glucose, and that any slight increase in the blood sugar following benzoate would then lead to the appearance of glucose in the urine. That this was not the case is shown by the blood sugar curves taken after glucose (Figs. 1 and 2). As mentioned earlier, no trace of glucose was found in the case of dog "T", but in dog "W", although the blood sugar rose to 196 mg. p.c., only traces of glucose were present in the samples of urine obtained some 12 hours after the test. In addition, experiments were carried out to determine the effect of sodium benzoate on the level of the blood sugar. Although a slight rise was obtained in both animals, this was also obtained in the case of untreated ones, and appears to be due to the handling which is necessary, since rises of similar magnitude occur in the blood sugar of these animals when sodium benzoate has not been administered.

Although the results concerning the effect of anterior pituitary extract on the elimination of benzoic acid were obtained from studies on only two dogs, they must be regarded as significant. In support of this contention the following facts are advanced:

(1) Following the administration of sodium benzoate to nine normal dogs, not injected with anterior pituitary extracts, no fermentable reducing substance could be detected in the urine. (2) In dog "T" sodium benzoate was administered eight times whilst the animal was receiving anterior pituitary extract, and on every occasion the phenomenon described above was reproduced. (3) At the moment of writing the administration of anterior pituitary extract to dog "T" has been discontinued for approximately 150 days. The glucose tolerance curve and the blood sugar response after insulin are now normal and, in addition, the elimination of sodium benzoate follows the course observed in normal animals. No evidence is at present available to account for the phenomenon described, but work is in progress to ascertain the mechanisms involved.

SUMMARY.

The detoxication of benzoate has been investigated in two dogs after treatment with injections of anterior pituitary gland.

The appearance of benzoyl-glycuronic acid in the urine of these animals was accompanied by a marked excretion of glucose.

ACKNOWLEDGMENTS.

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REFERENCES.

Brakefield, J. L. (1927): J. biol. Chem., 74, p. 783.
Quick, A. J. (1926): *Ibid.*, 70, p. 59.
Quick, A. J. (1932): *Ibid.*, 95, p. 189.
Young, F. G. (1939): New Eng. J. Med., 221, p. 635.

THE INFLUENCE OF FRUCTOSE UPON THE PERIPHERAL UTILISATION OF GLUCOSE

by

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Recently Fletcher and Waters (1938) have described some interesting experiments concerning the influence of fructose on the glucose tolerance curve. Davidson *et al.* (1936) showed that the intravenous injection of fructose depresses the glucose tolerance curve in rabbits, and Fletcher and Waters have confirmed this effect in dogs. Furthermore, they have shown that the phenomenon occurs in the depancreatized animal, so that the increased glucose tolerance after fructose cannot be attributed, as Davidson suggested, to an increased output of insulin. In discussing the increased utilization of glucose that occurs after fructose administration, Fletcher and Waters consider that this occurs chiefly in the liver where glycogenesis is accelerated. In the course of some earlier experiments (Corkill and Nelson, 1939), it was observed that fructose appeared to accelerate the fall in blood glucose produced by insulin in the eviscerated cat preparation. Hence it was thought worth while to obtain more precise information as to whether fructose caused an accelerated utilization of glucose by the peripheral tissues.

METHODS.

Practically the same experimental procedures described in the previous paper were followed. The spinal eviscerated cat preparation was given a continuous intravenous infusion of glucose plus fructose, or, in the case of control animals, glucose alone. In the latter instances the sugar was infused at the rate of 0·12 gm. per kg. body weight per hour. When glucose plus fructose was infused the rate and amount of glucose were the same as in the control experiments, whilst fructose was infused at from 0·09 gm. to 0·12 gm. per kg. per hour. Cats of approximately the same weight were used and after allowing one hour to elapse after evisceration, the intravenous infusion was commenced and allowed to continue for one and one-half hours, by which time the blood glucose usually has reached a steady level. Following preliminary blood glucose estimations, ten units of insulin were injected into the femoral vein and blood samples taken at half-hourly intervals for a period of two hours. Control experiments were made in which only glucose was infused. Blood glucose and blood fructose estimations were carried out by the methods previously described.

RESULTS.

In Table 1 the results of the above described experiments are shown, and it will be observed that the fall in blood glucose is markedly increased by fructose. The final values for blood glucose were always much lower in the presence of fructose, and when one makes allowance for non-fermentable reducing substances, it would appear that in some instances the glucose content is negligible.

TABLE 1.

Control Animals. Intravenous Infusion of Glucose. Blood Glucose in mg. p.c.				Test Animals. Intravenous Infusion of Glucose + Fructose. Blood Glucose in mg p.c.			
Cat Weight kg.	Initial	2 hours after insulin.	Decrease.	Cat Weight kg.	Initial.	2 hours after insulin.	Decrease.
3·5	331	176	155	3·3	320	19	301
3·7	318	173	135	3·6	280	6	274
2·8	210	50	160	2·9	330	25	305
3·2	298	147	151	3·0	300	6	294
3·4	327	183	144	3·4	297	4	293
3·5	340	190	150	3·7	333	6	327

The accelerating effect of fructose on glucose utilization is well shown by the curves in Fig. 1. These curves were obtained on spinal eviscerated cats of approximately the same weight.

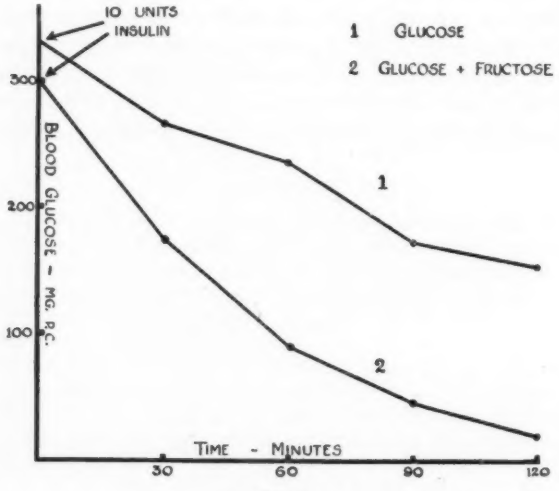

Fig. 1.

The same phenomenon can also be demonstrated after an intravenous injection of fructose.

Experiment:

Spinal eviscerated cat preparations. Intravenous infusion of glucose at the rate of 1·2 gm. per kg. of body weight per hour.

TABLE 2.

Cat weight. kg.	Blood glucose. mg. p.c.	10 units insulin + 1·6 gm. fructose. injected into femoral vein.	Blood glucose 2 hours after insulin injection. mg. p.c.
3·3	280	↓	6
3·0	320		Less than 4·5

Comparing these results with the control values in Table 1, the stimulating effect of fructose on glucose utilization is again evident.

There is one point that calls for comment in the above series of experiments, namely, the extent to which the liver contributes to the blood glucose. In their experiments on the eviscerated cat, Best *et al.*, realized that the liver exists as a cul-de-sac organ over the inferior vena cava and, whilst not taking any active part in the metabolic changes produced by insulin, can nevertheless contribute glucose to the circulation. Accordingly, when constructing their balance sheet for glucose disappearing and glucose found, the above factor was taken into account. It might be argued that, if wide variations occurred in the amount of glucose contributed by the liver, the present results would give erroneous conclusions, since all possible factors were not considered. However, from previous experiments we can state that such extreme variations in added liver glucose do not occur and, furthermore, it would be extremely improbable that in the control experiments the added glucose from the liver would invariably be greater than in the experiments where both glucose and fructose were infused.

Although we consider that our results indicate that fructose increases the peripheral utilization of glucose, we do not wish to imply that there is not also a similar effect in respect of hepatic glycogenesis. In some further experiments we endeavoured to demonstrate this latter phenomenon.

Experiment:

Young rabbits from the same litter and from 6 to 8 weeks old were deprived of food for twenty-four hours. The weights of the animals varied from 800–900 gm. The experimental details and combined results from several experiments are shown in Table 3.

TABLE 3.

	Normal fasting controls.	Given glucose orally, 1·5 gm. per kg. body-weight Killed 2 hours later.	Given 0·5 gm. fructose per kg. body-weight intravenously. Killed 1½ hrs. later.	Glucose 1·5 gm. per kg. body-weight orally; 30 mins. later. 0·5 gm. fructose per kg. intravenously. Killed 1½ hrs. after fructose.
		Liver glycogen per cent.		
	0·85	2·8	1·3	3·5
	0·78	2·4	1·4	2·9
	0·82	2·9	1·2	2·5
	0·60	3·0	1·1	2·2
	0·40	2·0	0·9	2·3
	0·34	2·8	0·8	3·1
	0·30	2·6	1·1	2·4
	0·28	2·7	1·2	3·9
Average:	0·54	2·6	1·1	2·8

Taking these results in relation to each other there is no evidence that, under the above experimental conditions, fructose increases hepatic glycogenesis.

In view of the difficulty in adjusting the experimental conditions to demonstrate a possible effect of fructose on glycogenesis, we must not conclude from the above results that this phenomenon does not occur. Again, it is possible that an effect in this direction, whilst producing a demonstrable change in the glucose tolerance curve, may nevertheless not be revealed by studies on liver glycogen.

SUMMARY.

1. Blood glucose estimations have been carried out on the spinal eviscerated cat receiving a continuous infusion of glucose, and it has been shown that fructose greatly increases the fall in blood glucose produced by insulin.

2. It is apparent from the results presented that fructose increases the peripheral utilization of glucose.

3. Although no definite evidence is available, it is possible that fructose also stimulates hepatic glycogenesis.

REFERENCES.

Corkill, A. B. and Nelson, J. F. (1939): Austral. J. exp. Biol., 17, p. 205.
Davidson, J. N., Kermack, W. O., Mowat, D. M. and Stewart, C. P. (1936): Biochem. J., 30, p. 433.
Fletcher, J. P. and Waters, E. T. (1938): Biochem. J., 32, p. 212.

THE ESTIMATION OF SULPHANILAMIDES AND OTHER PRIMARY AROMATIC AMINES IN BODY FLUIDS

by

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(Submitted for publication 17th February, 1940.)

Experiments on laboratory animals indicate that the therapeutic effects produced by drugs of the sulphanilamide group are primarily due to the bacteriostatic action of such compounds. King *et al.* (1939) have shown, in experiments with tissue cultures, that the bacteriostatic action varies directly with the concentration of the drug. Hence, to obtain the best therapeutic results with sulphanilamides, it would appear necessary to carry out blood analyses in order to ensure that an adequate concentration of the particular compound exists in the blood stream. The results of several investigators, namely, Marshall (1938), Baines and Wien (1939) and Stockinger (1939), show that, after oral administration, sulphanilamide compounds occur in the blood and urine partly as such and partly as acetylated derivatives.

During the course of a clinical investigation at this Hospital, the laboratory was requested to carry out blood sulphanilamide estimations. Since a large number of tests was required over short periods, it was desirable to select a quick yet accurate method, and the technique recently described by Werner (1939) seemed most suitable. In this method the serum proteins are precipitated with trichloracetic acid. After filtration, NaOH is added and the solution is then allowed to react with Ehrlich's reagent. Colorimetric determination gives free sulphanilamide, but the estimation of bound sulphanilamide necessitates hydrolysis of the acetyl compound to set free the aromatic amino group and, as a hydrolysing agent, Werner utilizes the trichloracetic acid that is already present in the blood filtrate. After hydrolysis, NaOH is added in the same quantities as in the estimation of the free form and, after mixing with Ehrlich's reagent, colorimetric readings are taken.

Apart from a point that will be discussed later, the estimation of free sulphanilamide presents no difficulties, but the method for combined sulphanilamide is open to serious criticism. Apparently Werner has not realized that trichloracetic acid is destroyed by heating in boiling water, and at the end of the hydrolysis period about 80 p.c. will have disappeared. Hence, after the addition of NaOH, the hydrolysate, instead of having the same acidity as the filtrate in which the free

form was estimated, will be less acid. It is found that with the same amount of sulphanilamide a greater intensity of colour will be produced with decreased acidity. The significance of this fact is obvious. Thus, if the hydrolysate is less acid than the filtrate in which the free form was estimated, the colour developed will give fallacious values for bound sulphanilamide.

This is well illustrated by the figures shown in Table 1. Estimations were made on oxalated whole blood taken from patients receiving *M. and B. 693* (2-sulphanilamidopyridine). The first two columns show the values obtained by Werner's method for free and total sulphanilamide respectively. The values shown in column three for total sulphanilamide were obtained after neutralization of the hydrolysate. It is not to be inferred from these results that combined sulphanilamide does not exist in the blood. It will be shown later that considerable individual variations occur in the time of appearance of the acetyl derivative in the blood stream. In most of the above cases conjugation had not occurred, although Werner's method would have led one to believe that such was the case.

TABLE 1.

Estimation of Free and "Total" Sulphanilamide in Oxalated Whole Blood after the Administration of M. and B. 693.

Results are given in mg. *M. & B. 693* per 10 ml. blood.

Free.	Total (1).	Total (2).
4·6	5·2	—
7·8	9·2	—
1·2	1·5	1·0
1·8	2·2	1·5
1·9	2·5	2·0
2·2	3·4	2·8
1·0	1·6	1·2

The above points are further illustrated in Fig. 1. Werner's reaction for combined sulphanilamide was applied to an aqueous solution of *M. and B. 693*. The extinction coefficients of the resultant coloured solution were determined in the Lange photo-electric colorimeter with blue filters having a transmission of 4050 to 4800Å.

Curve 1 shows the values obtained with the unmodified method of Werner, whilst Curve 2 gives the values obtained after the addition of 0·5 ml. of 2 p.c. NaOH to the solution.

A further criticism can be made on the grounds that Ehrlich's reagent gives a yellow colour with urea. In normal individuals with a blood urea in the vicinity of 40 mg. p.c., the colour produced corresponds to 0·2 to 0·4 mg. p.c. *M. and B. 693*. Hence, in the estimation of sulphanilamide, an error will be present even in normal individuals, and with small concentrations of the drug this cannot be neglected. Greater discrepancies will naturally occur in patients with urea retention.

Although Werner's method gives erroneous results for the total sulphanila-

mide, the free form, in patients with a normal blood urea, can be rapidly determined as follows:

1 ml. of oxalated blood or serum and 4 ml. of 5 p.c. trichloracetic acid are mixed in a test tube and thoroughly stirred with a glass rod. After filtration, 2 ml. of the filtrate are measured into a tube graduated at the 4 ml. mark, and a small drop of a 0·25 p.c. alcoholic solution of phenolphthalein is added. The solution is then neutralized to a faint pink colour with 2 p.c. NaOH; 0·5 ml. of Ehrlich's aldehyde reagent (3 gm. of *p*-dimethylamidobenzaldehyde dissolved in 100 ml. 14 p.c. H_2SO_4) is added and the volume made up to 4 ml. with distilled water. After standing for 10 minutes the solution is compared with K_2CrO_4 standards. A set of standard chromate solutions is prepared, starting from 0·01 p.c. K_2CrO_4 which gives a colour closely corresponding to 1·4 mg. p.c. of *M. & B. 693*. It must be noted that this relationship will not be applicable to other sulphanilamide derivatives and, in order to determine these, new values must be given to the original set of chromate standards.

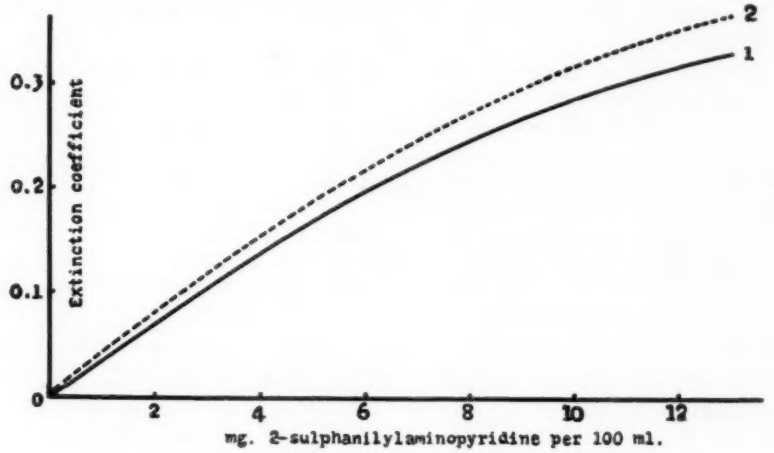

Fig. 1. For explanation of curves see text.

Probably the most sensitive methods that have been introduced for the determination of sulphanilamide are those that depend upon diazotization and linkage of the diazo compound with a reagent that will produce a dye. For this latter purpose Marshall (1938) and Marshall and Litchfield (1938) have recommended dimethyl α-naphthylamine, which gives a purple-coloured compound with aromatic diazo substances. Unfortunately some difficulty was found in obtaining samples of the amine that will fulfil the required conditions, namely, a good speed of colour development and a small colour blank. α-naphthol, which, in alkaline solution, gives red-coloured azo dyes with diazotized aromatic amines in high dilutions, was then tried. At the start diazotization was made in the presence of *p*-toluene sulphonic acid, since Marshall has shown that the latter can be used for preliminary deproteinization and then for hydrolysis of conjugated sulphanilamide in the protein-free filtrate.

Varying amounts of primary aromatic amines were dissolved in water, *p*-toluene sulphonic acid was added to give a final concentration of 4·5 p.c. of this acid, and coupled with α-naphthol after the technique to be described later. The results of these experiments are shown in Table 2.

TABLE 2.

		Extinction coefficient.	
	Mg. p.c.	Before heating.	After heating for 90 min. in the water-bath.
Sulphanilic acid	16·2	0·042	0·013
" "	8·1	0·009	0·004
Sulphanilamide	1·45	0·092	0·076
"	0·72	0·046	0·031

These figures show that it is not practical to use *p*-toluene sulphonic acid for sulphanilamide determinations, since heating in the water-bath causes a loss of sulphanilamide.

However, if trichloracetic acid is used for the deproteinization and HCl is added in quantities that would compensate for the loss of trichloracetic acid during hydrolysis, satisfactory results can be obtained. The values shown in Table 3 indicate that these aromatic amines may be heated for two hours in the presence of trichloracetic acid plus HCl without any loss.

TABLE 3.

		Extinction coefficients after diazotization and coupling with α-naphthol.	
Substance used.	Mg. p.c.	Solutions containing 4 p.c. trichloracetic acid.	The same solutions, plus 1 ml. 4·5 p.c. HCl, and heating for 2 hours in the water-bath.
Sulphanilic acid*	3·92	0·251	0·248
	2·61	0·166	0·170
	1·30	0·090	0·084
	0·52	0·036	0·033
Sulphanilamide*	4·57	0·284	0·281
	2·28	0·152	0·151
	1·14	0·078	0·076
	0·23	0·014	0·014
*M. & B. 693**	5·10	0·243	0·241
	2·55	0·128	0·127
	1·28	0·065	0·063
	0·51	0·027	0·028
	0·26	0·013	0·013
	0·13	0·007	0·006

* The above compounds were obtained in the commercial form and then recrystallized from water and dried in vacuo over P_2O_5 at 100°C.

The technique recommended for the estimation of free amino compounds in body fluids is as follows:

To 2 ml. of whole blood, serum or cerebrospinal fluid are added 14 ml. distilled water and 4 ml. 20 p.c. trichloracetic acid (weight-volume). After shaking thoroughly, the mixture is

filtered. To 4 ml. of filtrate is added 0·4 ml. of a freshly prepared 0·5 p.c. solution of $NaNO_2$. The reaction is allowed to proceed for 5 minutes at a temperature of about 10°C. Finally, 2 ml. of N. NaOH are added and immediately afterwards 0·5 ml. of 0·5 p.c. α-naphthol in methylated spirits. Thorough mixing after each addition is important. After allowing to stand at room temperature for 15 minutes, readings are taken in a photo-electric colorimeter with filters having a transmission of 4800–5460Å, and the results referred to a standard curve.

Table 4 shows the values found when these compounds are added to normal human serum.

TABLE 4.

Recoveries of Sulphanilamide Added to Normal Serum.

	Added. Mg. p.c.	Found. Mg. p.c.
M. & B. 693	5·1	4·9
	2·55	2·3
	1·02	0·92
Sulphanilamide	1·82	1·80
	0·45	0·43
	0·09	0·08

For clinical purposes it has been found that the Lovibond tintometer with a bilirubin disc affords a convenient means of comparison. The numbers on the disc multiplied by 2·6 give mg. p.c. *M. and B. 693*. The readings can be simplified by using aqueous solutions of cobalt chloride. A solution of 0·25 p.c. $CoCl_2$, $7H_2O$ has a colour corresponding approximately to 0·64 mg. p.c. *M. and B. 693*.

The limit for the determination of free sulphanilamide compounds in a 1/10 dilution of blood is about 0·1 mg. p.c., this concentration giving quite a distinct colour. A comparison between the *p*-dimethylamidobenzaldehyde and the α-naphthol reactions, in sera with normal blood urea content, gives, as shown in Table 5, quite a close agreement.

TABLE 5.

Reaction with *p*-dimethylamidobenzaldehyde. Mg. p.c. *M. & B. 693*.	Reaction with α-naphthol. Mg. p.c. *M. & B. 693*.
1·6	1·5
1·7	1·75
2·0	1·86
2·4	2·3
2·9	3·05
3·6	3·5
2·6	2·7
0·8	0·85

For the determination of the free amino compounds in the urine, the latter must be diluted at least 100 times with distilled water. From this dilution 3·2 ml. are taken and 0·8 ml. of 20 p.c. trichloracetic acid is added. This solution is diazotized and coupled with α-naphthol, as previously described.

Estimation of Combined Sulphanilamides.

As already discussed, a simple hydrolysis with trichloracetic acid, as recommended by Werner and van der Zoo de Jong (1939), does not give satisfactory results, but, as is shown in Table 6, with a concentration of 0·25 N. to 0·3 N. HCl in the trichloracetic acid solution, approximately 95 p.c. of acetylated *M. and B. 693* can be recovered from aqueous solution. Baines and Wien (1939) have proposed an alkaline hydrolysis and, using this method, good recoveries have been obtained; but as in this process silica is dissolved even from pyrex glass, its subsequent removal by filtration is a disadvantage.

TABLE 6.

Estimation of Acetylsulphanilamidopyridine in Aqueous Solution. Hydrolysis with Trichloracetic Acid + HCl.

Acetylated *M. & B. 693.* Mg. p.c.	*M. & B. 693* Found Mg. p.c.	Calculated Mg. p.c.
14·7	12·1	12·5
11·0	8·8	9·4
7·35	5·9	6·3
3·67	2·9	3·14
1·47	1·2	1·26

Although, as can be seen in Table 6, good recoveries are obtained from aqueous solutions, such is not the case when acetyl *M. and B. 693* is added to normal serum. Here the maximum amount recovered is about 85 p.c. Marshall (1938) and Scudi (1938) have discussed this loss of acetylated sulphanilamide added to serum, and Marshall suggests that it may be overcome by further dilution of the serum prior to deproteinization. Experiments carried out along these lines do not support Marshall's contention that preliminary dilution of serum enables added acetyl sulphanilamide to be satisfactorily recovered. From Table 7 it will be seen that there is still a loss of approximately 15 p.c.

TABLE 7.

Estimation of Acetylsulphanilamidopyridine Added to Normal Serum.

Acetylated *M. & B. 693.* Mg. p.c.	*M. & B. 693* Found (Serum dilution 1/10). Mg. p.c.	Found (Serum dilution 1/20). Mg. p.c.	Calculated Mg. p.c.
5·05	3·56	3·64	4·32
2·52	1·84	1·88	2·16
1·26	0·92	0·92	1·08
0·5	0·36	0·32	0·43

For the estimation of bound sulphanilamide in body fluids the following procedure has been adopted:

To 4 ml. of the trichloracetic acid filtrate add 1 ml. of 4·5 p.c. HCl and mix in a test tube graduated at 4 ml. The hydrolysis is made by heating for 2 hours in boiling water. After cooling

to room temperature, the volume is made up to 4 ml. with water and the diazotization and coupling with α-naphthol performed as described for the free form. Urines are diluted at least 200 times with water, and to 3·2 ml. of the dilution are added 0·8 ml. of 20 p.c. trichloracetic acid and 1 ml. of 4·5 p.c. HCl, and then the procedure follows that described for blood.

It must be noted that the determinations of *M. and B. 693* should be done in serum, since oxalated plasma gives lower results. Whole blood (see Table 10) gives far lower results than serum, owing to the small content of *M. and B. 693* in the corpuscles.

It has been found that with the oral administration of sulphanilamide considerable individual variations occur in the time of appearance of the conjugated form in the blood stream. At present it is uncertain whether such differences are dependent upon the conjugating power of the liver or upon a decreased renal threshold for the acetyl derivative. Several workers have shown that conjugated sulphanilamide is cleared from whole blood far more rapidly than the free form.

TABLE 8.

Sulphanilamide Estimations on Three Normal Individuals.

Time of collection after administration of *M. & B. 693.*	I				II		III	
	Serum. Mg. p.c. *M. & B. 693.*		Urine. Mg. p.c. *M. & B. 693.*		Serum. Mg. p.c. *M. & B. 693.*		Serum. Mg. p.c. *M. & B. 693.*	
hr.	Free.	Total.	Free.	Total.	Free.	Total.	Free	Total.
½	—	—	0	0	—	—	—	—
1	—	—	—	—	2·5	2·6	3·2	3·5
2	2·5	2·9	20·8	33·2	2·9	2·8	4·8	4·8
3	2·8	3·6	—	—	2·8	3·0	6·1	6·1
4	3·2	4·2	68·0	130·0	2·7	2·9	6·2	6·2
5	2·9	4·4	—	—	2·5	2·6	5·9	6·2
7	2·5	4·1	84·0	220·0	2·3	2·5	5·1	5·2
9	2·3	3·8	33·0	84·0	2·3	2·6	4·4	4·6
12	1·8	3·2	40·0	118·0	2·3	2·6	4·0	4·6
24	1·1	1·9	43·0	200·0	1·6	2·0	2·3	3·1

Table 8 shows the results of progressive blood examinations for free and bound sulphanilamide on three normal individuals who received 3 gm. of *M. and B. 693*. In addition, urinary estimations on patient No. 1 are included. In the first individual, evidence of conjugation appears after the third hour; but in Nos. II and III no significant effect is seen until the twenty-fourth hour. Although the blood values in patient No. I are not striking, the figures for the urine indicate that active conjugation has occurred.

By further experiments it was shown that the pH of the urine had an influence on the excretion of the conjugated form. This was demonstrated in a series of experiments with mice.

Two groups, each consisting of five mice whose body weights were 20 gm., were treated in the following manner:

Group I. Each mouse received 1 ml. of an aqueous suspension of 100 mg. sulphanilamide orally in two doses with a 5-hour interval between. This amount of sulphanilamide is very close to the lethal dose.

Group II. 1 ml. of 3 p.c. Na_2CO_3 solution suspended with 100 mg. sulphanilamide was administered as in Group I.

Both groups were put in metabolism cages with a liberal supply of bread and water. The animals of Group I exhibited marked symptoms of distress shortly after treatment and one died after three hours, whilst the remaining four recovered after 24 hours. In contrast to this the mice of Group II, apart from diarrhoea, showed no toxic symptoms, although one died after 30 hours. The 24-hour specimens of urine obtained from these mice were examined and no combined form could be detected in either group. Since mice are known to acetylate sulphanilamide, it was thought that the amount given was too close to the lethal dose and consequently another experiment was conducted, as shown below.

Group III. Five mice (each 20 gm. weight) were fed with 0·5 ml. of an aqueous suspension containing 10 mg. sulphanilamide.

Group IV. Five mice were fed with 0·5 ml. of 2 p.c. Na_2CO_3 containing 10 mg. sulphanilamide.

In both cases the sulphanilamide was given in a single dose. In no case did the animals show any toxic symptoms. The urines were collected 5 and 24 hours after feeding. The results of these experiments are shown in Table 9.

TABLE 9.

Group.	Urine.				Sulphanilamide in mg.			
	After 5 hrs.		After 24 hrs.		After 5 hrs.		After 24 hrs.	
	ml.	pH.	ml.	pH.	Free.	Bound.	Free.	Bound.
I	—	—	7·0	8·0	—	—	96·4	Nil
II	—	—	5·5	8·4	—	—	66·4	Nil
III	4·0	7·8	10·35	6·8	8·1	Nil	18·04	2·94
IV	2·5	8·4	9·06	8·0	8·09	Nil	23·39	6·93

From the above table it can be seen that the control mice excreted 36 p.c. of the administered sulphanilamide in the free form and 6 p.c. of the combined form in 24 hours, the ratio of the free to the acetylated form being 6 : 1. On the other hand the mice fed with the alkaline suspension excreted 47 p.c. of the free and 14 p.c. of the combined form in 24 hours, the ratio in this case being 3·4 : 1. The increased excretion of the acetyl sulphanilamide in the case of the animals treated with Na_2CO_3 suggests that alkalis might be of some clinical value in preventing the undesirable effects of drugs of the sulphanilamide series.

Distribution of Sulphanilamidopyridine between Serum and Corpuscles.

The distribution of sulphanilamide compounds between the blood plasma and the blood cells is of interest, firstly, from a practical point of view, as to whether whole blood or plasma should be taken for the quantitative determination of the drug and, secondly, from a theoretical point of view, in respect of the question of the bactericidal power of plasma and cells. As previously mentioned, there is usually a higher concentration of *M. and B. 693*, both free and combined, in the serum than in whole blood. This indicates that there cannot be an even distribution in the whole blood. Baines and Wien (1939) found that all the free amino-body was contained in the serum. Their conclusions are drawn from experiments in rabbits to which *M. and B. 693* was given orally. Estimations of the amino-compound were made in the serum, whole blood and corpuscles. The latter were

obtained by repeated washings with saline solution in order to free them from plasma. No free amino compound was found in the corpuscles but, in spite of the conclusions of these authors, a consideration of their published results shows that there is very close agreement between the amount of *M. and B. 693* in whole blood and serum, thus indicating an even distribution between the plasma and cells in whole blood.

My own experiments were made on human and rabbit blood. Blood was collected from a vein without the addition of an anti-coagulant, because it was found that potassium oxalate diminishes the intensity of colour produced after diazotization and coupling. Usually 2 ml. of whole blood were added to 14 ml. of distilled water, and after haemolysis 4 ml. of 20 p.c. trichloracetic acid were added and the estimation carried out as previously described. Another portion of the whole blood was allowed to clot and the determination was made in the cell-free serum. Lastly, a third portion was mixed with powdered potassium oxalate and the estimation of the corpuscular volume made with the haematocrit. From the figures obtained the amount of the drug in the corpuscles was calculated. The calculations show that the corpuscles appear to contain in every case an appreciable amount of free *M. and B. 693*. In the case indicated by an asterisk a suspension of corpuscles was prepared by washing the whole blood with 0·85 p.c. NaCl solution and no amino compound was detected by the above method. Hence it was assumed that the amino-body diffused out during washing. In addition to the estimation of the free amino compound, determinations of the conjugated form have been carried out, and the results obtained indicate that the combined form of *M. and B. 693* exists only in the plasma. To investigate this question experiments were made on human and rabbits' blood, and the results are shown in Table 10.

TABLE 10.

Distribution of M. and B. 693 *between Serum and Corpuscles.*

(Figures are given in mg. *M. & B. 693* per 10 ml.)

	Whole blood.		Serum.			Oxalated plasma.	Corpuscle volume.	Calculated in the corpuscles.	
Patient	Free.	Total.	Free.	Total.	Free.	Total.	p.c.	Free.	Bound.
A	5·8	6·1	7·6	10·2	7·0	9·44	46·4	3·7	0
B	3·2	4·3	4·76	7·8	4·2	6·7	46·0	1·4	0
C	3·5	5·6	4·8	9·2	—	—	33·0	0·9	0
D	3·0	2·9	3·6	3·6	—	—	33·0	1·8*	0
Rabbit									
I	6·7	12·4	9·4	20·4	—	—	34·6	1·6	0
II	4·4	9·6	5·9	14·7	—	—	34·2	1·5	0

The rabbits received 0·5 gm. *M. & B. 693* per kg. body weight by stomach tube. Blood was taken from the ear vein 4 hours later.

Hansen (1939) and Sise (1939) have shown that in the case of sulphanilamide there is always a higher concentration in whole blood than in serum, and it is puzzling that there should be a different behaviour in the case of the allied sulphanilamidopyridine.

SUMMARY.

The reaction between sulphanilamide and *p*-dimethylamidobenzaldehyde in serum filtrates has been investigated. Free sulphanilamide in blood can be rapidly determined by Werner's method.

A new method has been developed for the estimation of primary aromatic amines in body fluids, employing a diazotization and subsequent coupling of the diazo compound with α-naphthol in alkaline solution. This reaction can be applied for derivatives of those amines which, after hydrolysis, yield the free amino group.

Estimations in sera of patients undergoing treatment with *M. and B. 693* show that the ratio between free and total *M. and B. 693* varies in different individuals. Whether such differences can be explained by the variations in the conjugating power of the liver or by the differences in the clearance rates cannot be decided at the moment, but it appears, if deductions from experiments made with mice are permitted, that food has an influence on the excretion. It was observed that animals on a diet producing an alkaline urine excreted greater amounts of the acetylated *M. and B. 693*.

Studies on the distribution of the free *M. and B. 693* in humans and rabbits who received *M. and B. 693* orally indicate that this particular drug is found in a higher concentration in the serum than in the whole blood. Indirect determinations indicate that between 20 and 50 p.c. of the free form is contained in the corpuscles. On the other hand, direct estimations by other workers on washed corpuscles show that there is no free amino body present. These results would seem to indicate that *M. and B. 693* in the corpuscles is lost by a process of diffusion during the washing.

Sulphanilamidopyridine, unlike sulphanilamide, is found in a higher concentration in the blood serum than in the whole blood. The corpuscles contain smaller amounts of *M. and B. 693* than the serum.

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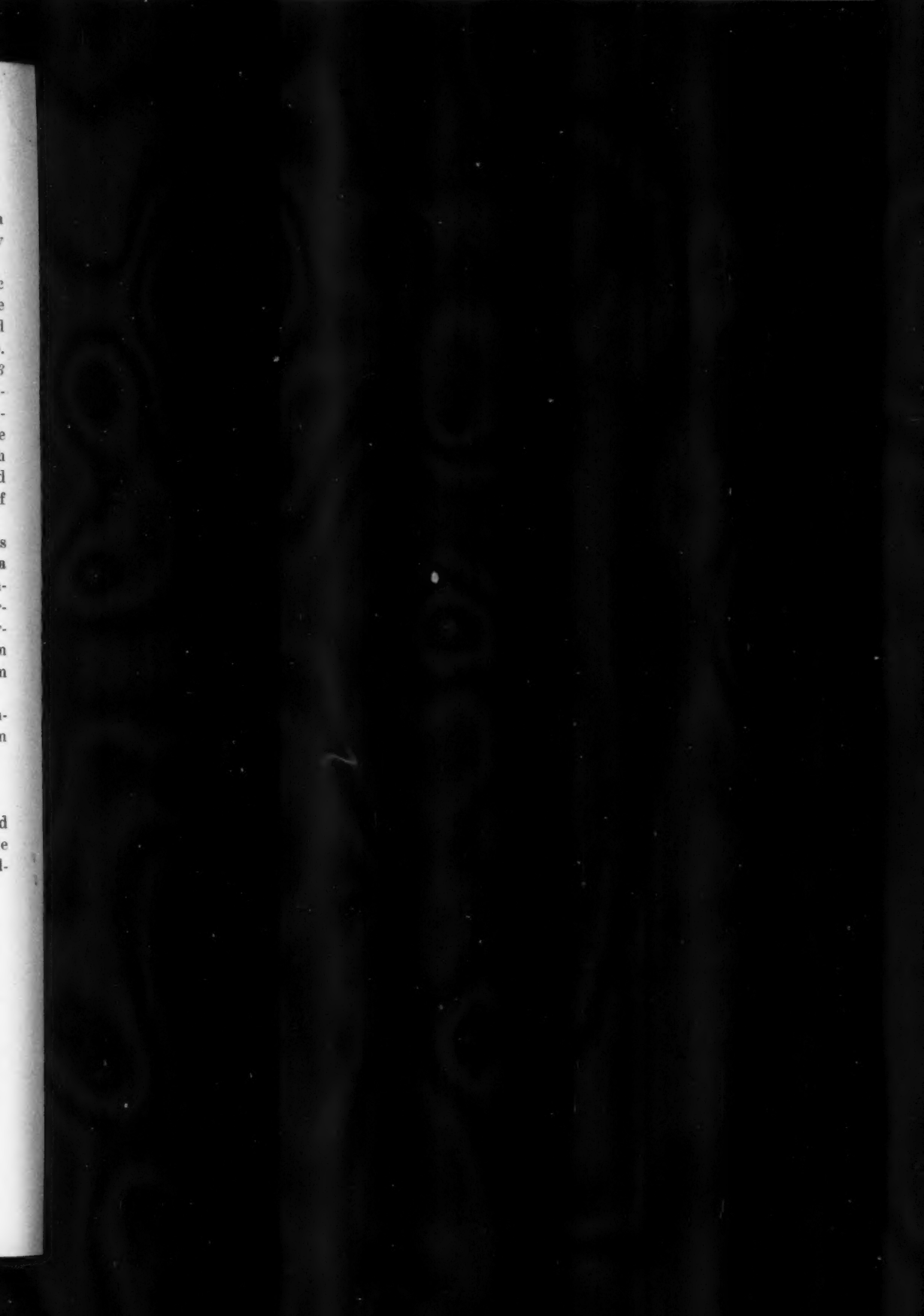
REFERENCES.

Baines, E. J. and Wien, R. (1939): Quart. J. Pharm. and Pharmacol., 12, p. 4.
Hansen, L. (1939): J. Pharmacol. & exp. Therap., 65, p. 372.
King, J. T., Henschel, A. F. and Green, B. S. (1939): J. Amer. Med. Assoc., 113, p. 1704.
Marshall, E. K., junr. (1938): J. biol. Chem., 122, p. 263.
Marshall, E. K., junr. and Litchfield, J. T., junr. (1938): Science, 88, p. 85.
Scudi, J. V. (1938): J. biol. Chem., 122, p. 539.
Sise, H. S. (1939): Proc. Soc. exp. Biol. & Med., 40, p. 451.
Stockinger, H. E. (1939): *Ibid.*, 40, p. 61.
van der Zoo de Jong, H. (1939): Lancet, 2, p. 833.
Werner, A. E. A. (1939): *Ibid.*, 1, p. 18.